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CONTENTS

No. 1 (January, 1939)

Original Articles: PAGE

205. Methods of measuring gas production. L. A. Allen . 1

206. Gas production by *Bacterium coli* and by lactic acid streptococci under different conditions. M. M. Hassouna and L. A. Allen 7

207. Protein metabolism and acid production by the lactic acid bacteria in milk. Influence of yeast extract and chalk. M. Braz and L. A. Allen 20

208. The part played by bacteria in the reduction of methylene blue in milk. B. C. Hobbs 35

209. Studies in mastitis. I. The routine diagnosis of mastitis. J. G. Davis, J. McClemont and H. J. Rogers . . 59

210. Studies in mastitis. II. A comparison of methods for the detection of subclinical mastitis. J. G. Davis and J. McClemont 74

211. Studies in mastitis. III. Mastitis in relation to "udder counts". J. McClemont and J. G. Davis . . . 81

212. Studies in mastitis. IV. Mastitis in relation to the methylene blue reduction test. J. McClemont and J. G. Davis 88

213. Studies in mastitis. V. Mastitis in relation to cheese-making. J. G. Davis and J. McClemont . . . 94

214. Studies in mastitis. VI. General observations, summary and conclusions. J. G. Davis and J. McClemont . . 104

215. The value of indirect methods for the detection of mastitis. S. J. Rowland and M. Zein-El-Dine 108

216. The effect of churning on the vitamin A and carotene content of milk fat. K. M. Henry, S. K. Kon, A. E. Gillam and P. White 114

217. The white particles in mature Cheddar cheese. F. H. McDowall and A. K. R. McDowell 118

218. Starter cultures for cheese manufacture. Maintenance of acid-producing activity in cultures of lactic streptococci. H. R. Whitehead and G. J. E. Hunter . . . 120

Biennial Reviews of the Progress of Dairy Science:

Section A. Physiology of dairy cattle. I. Reproduction and lactation. J. A. B. Smith 133

II. Nutrition. S. Morris 153

No. 2 (May, 1939)

Original Articles:

219. The nutritive value of proteins for milk production. S. Morris and S. C. Ray 165

220. The nutritional requirements of the lactic acid bacteria. J. G. Davis 186

221. The effect of serum and blood on the growth of lactic acid bacteria in milk. J. G. Davis 196

222. Bacteriological studies of spray-dried milk powder. A. A. Nichols 202

223. Bacteriological studies of canned milk products. A. A. Nichols 231

224. The decomposition of citric acid by *Betacoccus cremoris*. J. van Beynum and J. W. Pette 250

225. The casein number for diagnosis of mastitis. II. The effect of advanced lactation, and of storage and preservation of the milk sample. S. J. Rowland and M. Zein-El-Dine . 267

226. The effect of commercial drying and evaporation on the nutritive properties of milk. K. M. Henry, J. Houston, S. K. Kon and L. W. Osborne 272

227. The rapid determination of peroxide values for the fat in milk powders. J. A. B. Smith 294

228. Oxidation of the fat of butter during cold storage. W. J. Wiley 300

229. Bacteriological testing of butter. New and simplified routine methods. G. M. Moir and R. R. Russell . . 310

230. The relation of certain lactic acid bacteria to open texture in Cheddar cheese. I. R. Sherwood 326

231. The assessment of curd firmness prior to cutting. F. M. V. Coppen 336

232. A simple and accurate viscosimetric form of rennet test. C. W. King and E. M. Melville 340

Biennial Reviews of the Progress of Dairy Science:

Section F. Milk-borne diseases. J. SMITH 355

Section G. Genetics of Dairy Cattle. A. D. BUCHANAN SMITH . 370

No. 3 (SEPTEMBER, 1939)

Original Articles:

233. The effect of increased iodine feeding upon the iodine content of cows' milk. N. L. MATTHEWS, G. M. CURTIS and J. H. MEYER 395

234. Bacteriophage-organism relationships in the group of lactic streptococci. H. R. WHITEHEAD and G. J. E. HUNTER . 403

235. The enrichment of *aerogenes-cloacae* types in milk held at low temperatures: with observations on the relative rates of growth of *aerogenes-cloacae* and *B. coli* types in milk at different temperatures. J. F. MALCOLM . . . 410

236. The bacterial flora of New Zealand Cheddar cheese. I. R. SHERWOOD 420

237. Lactic acid bacteria in relation to cheese flavour. II. Observations on the inoculation of the milk employed in cheese manufacture with lactobacilli. I. R. SHERWOOD . 449

238. The effect of *p*H on growth and gas production by streptococci and lactobacilli. J. G. DAVIS and C. C. THIEL . 455

239. Examples of variation within pure cultures of *Streptococcus cremoris*. G. J. E. HUNTER 464

240. The effect of light on the vitamin C of milk in different containers. J. HOUSTON, S. K. KON and S. Y. THOMPSON 471

241. Kay and Graham's phosphatase test: (*a*) Modifications in technique; (*b*) Effect of bacterial growth. F. K. NEAVE . 474

242. A rapid phosphatase test: (*a*) Adaptation of Scharer's modification to pasteurizing conditions in Great Britain; (*b*) A study of the factors influencing the reliability of the test. R. ASCHAFFENBURG and F. K. NEAVE . . . 485

243. Factors affecting the solubility of milk powders. IV. The influence of speed and duration of stirring on solubility, with a description of a rapid method for solubility determinations. G. R. HOWAT, J. A. B. SMITH, R. WAITE and N. C. WRIGHT 498

Biennial Reviews of the Progress of Dairy Science:

Section B. Bacteriology and Mycology applied to dairying. A. T. R. MATTICK, E. R. HISCOX and J. G. DAVIS . . . 515

VOLUME X JANUARY, 1939 No. 1

576.809.3

205. METHODS OF MEASURING GAS PRODUCTION

BY L. A. ALLEN
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(With 4 Figures)

GAS production in dairy products is a subject of considerable importance, since it is responsible for certain defects in milk, cream, cheese and occasionally condensed milk. Four groups of micro-organisms are concerned, namely, yeasts, lactic acid bacteria, coliform bacteria and spore-forming anaerobes, and of these the first two produce carbon dioxide only while the last two produce a mixture of carbon dioxide and hydrogen (with some anaerobes there may be methane as well). Where both gases are evolved the effectiveness of the two as regards ability to produce effervescence or to develop pressure in a closed container or in a hard-pressed substance like cheese differs by virtue of the fact that carbon dioxide is soluble in water (its solubility depending on the pressure and *p*H of the medium) while hydrogen is insoluble. Until the medium in which such micro-organisms are growing becomes saturated with carbon dioxide the effective gas production is therefore due to hydrogen. On the other hand, in some media the hydrogen may be involved in secondary reactions with some of the constituents while in the nascent condition and will therefore not form gas.

Two examples of the influence of lactic acid bacteria on gas production are (1) development by some species of streptococci of a small quantity of carbon dioxide which may contribute to flavour in starters, and (2) the restraining effect of the rapid growth of starter organisms, in milk which is being ripened for cheese or butter manufacture, on the growth and gas production by coliform bacteria present as contaminants.

It may be remarked also that, according to the species of organism concerned, the amount of gas produced will depend on whether the conditions are aerobic, micro-aerophilic or anaerobic.

It is evident that, in order to understand the effect of gas production under various conditions of growth and environment, it is of importance to know (1) the rate at which gas may be developed by different micro-organisms growing in various media, including milk and its products, (2) the effect on this gas production of imposing strictly anaerobic conditions from the commencement of growth, (3) the relative influence of carbon dioxide and hydrogen if both gases are evolved, (4) the influence of lactic acid streptococci in inhibiting gas production by other micro-organisms when growing in the same

medium with them, and (5) the total amount of carbon dioxide evolved in the case of organisms producing no other gas.

With these considerations in mind the following methods have been evolved. Their application to some specific problems is described in a subsequent paper (Hassouna & Allen (2)).

Description of apparatus and methods

Capillary tube method

Cranston (1) described a method for measuring gas production by denitrifying bacteria in which the culture tube, immersed in a thermostat, was connected to a long capillary tube containing a mercury pellet. The pressure of the developed gas pushed the pellet along the capillary tube, and by calibrating the latter the volume of gas formed at intervals during growth could be calculated by measuring the distance through which the pellet had been moved. This method has been modified for use in the present investigation.

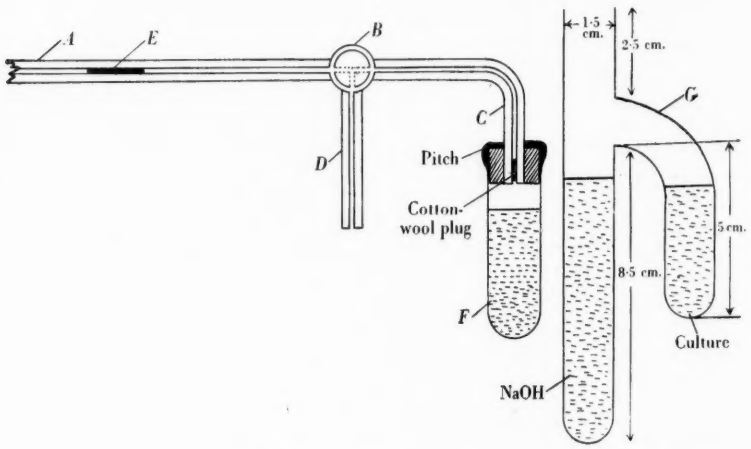

Fig. 1.

Fig. 2.

A capillary tube (*A*, Fig. 1), about 180 cm. long and 1·5 mm. bore, has one end *C* bent at right angles for a distance of about 3 cm. About 3 or 4 cm. from this end is a three-way glass tap *B* from which depends a short piece of tubing *D* (about 4 cm. long). A mercury pellet *E* is introduced into the capillary tube *A*, and the culture tube *F* is attached to the end *C*.

It is necessary that this capillary should be perfectly clean and dry before each experiment and that pure, dry, redistilled mercury should be used for the pellet so that movement of the latter should be sensitive to slight pressure and as even as possible. Cleaning is best accomplished by filling the tube with chromic acid-sulphuric acid mixture, leaving overnight, rinsing with dilute

alkali and finally washing thoroughly in distilled water, followed by alcohol and ether. The tube is dried finally by drawing warm air through it.

For working under aerobic conditions the tap *B* should be turned so as to connect *A* and *C*, while *D* should be disconnected and disregarded. A very slight turn of the tap will now disconnect all three tubes, *A*, *C* and *D*. If a suction pump is attached to the end *C* while the capillary tube is tilted so as to allow the end *A* to be below the surface of mercury contained in a small beaker, careful manipulation of the tap will enable mercury to be drawn slowly into the tube. When a pellet of sufficient length (about 1 in. is convenient) has been drawn in, the end *A* should be withdrawn from the mercury and the suction allowed to continue until the pellet has reached a suitable position (about 12 in. from the tap), when further motion is stopped by turning the tap slightly so as to stop suction. The pump is then disconnected and the tap *B* can be turned so as to connect *A* and *C*. The pellet will remain in the same position provided the capillary tube is kept reasonably horizontal.

The culture tube *F* (a convenient size is 5 cm. × 1·5 cm. internal diameter) is plugged with cotton-wool and sterilized by hot air while a one-holed rubber bung, which fits the mouth of the tube tightly, is sterilized separately in the autoclave. Into this culture tube are pipetted 5 ml. of the culture medium being investigated, together with a suitable inoculum of the test organism. The end *C* of the capillary tube is now flamed to sterilize it and a small plug of sterile cotton-wool inserted. The rubber bung is then fitted tightly over the tube *C* so that the end of the tube is flush with the lower end of the bung, and the latter is then pressed firmly into the culture tube. The dimensions of the latter are such that the air space above the culture fluid is small, so that development of gas is quickly registered by the pellet. If the bung fits tightly gas leakage will be negligible since practically no pressure is developed, but it is best to observe all precautions by running a layer of molten pitch over the bung and mouth of the tube, as shown in the diagram. The tap *B* should first be thoroughly cleaned with xylol, greased with a small quantity of tap grease, and held firmly in its socket by means of a rubber band.

If it is required to observe the effect of developed hydrogen, apart from carbon dioxide, the straight culture tube *F* is replaced by an h tube of the kind shown in Fig. 2 (*G*), which also gives the dimensions. The h tube is sterilized by hot air, 10 ml. of sterile 10 % sodium hydroxide pipetted into the left arm of the tube and 5 ml. of the culture fluid plus a suitable inoculum of the test organism into the right arm. The h tube is then fitted to the tube *C* as described for the culture tube *F* above.

For observing gas production under anaerobic conditions the space above the culture fluid in *F* (or *G*) and the portion of the capillary between the culture tube and the pellet *E* are filled with hydrogen before taking readings. For this purpose the mercury pellet is first drawn along the capillary by careful suction until it just touches the tap *B*. The latter is then turned so as to connect *C* and *D* only, thus insulating the pellet from the effect of changes

of pressure which would alter its position. After fitting the culture tube to *C* in the usual way the tube *D* is attached, through a glass tap *H*, to a four-way glass tube (as shown in Fig. 3), one arm of which is connected to a manometer. A second arm is connected to a hydrogen cylinder, the hydrogen being first passed through a wash bottle *J* containing alkaline pyrogallol (conveniently made by adding to the wash bottle just before use a mixture of sodium hydroxide solution of specific gravity 1·55 and a 10% solution of pyrogallol) in order to remove traces of oxygen. A glass tap *K* is interposed between the wash bottle and the hydrogen cylinder so as to allow of a very slow stream of gas. The remaining arm is connected, through a glass tap *L*, to a suction pump.

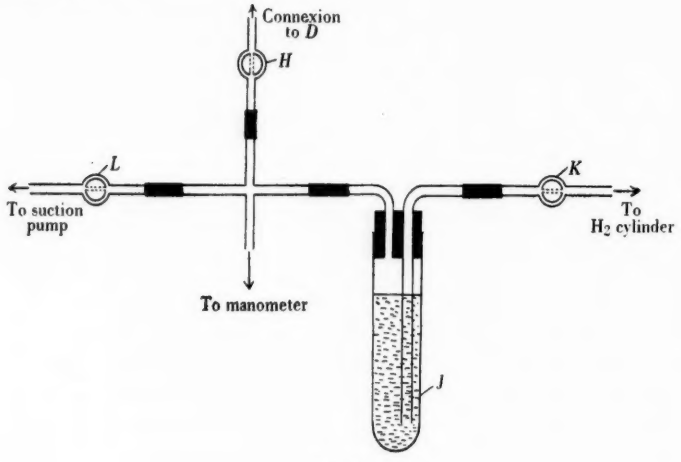

Fig. 3.

The taps *H* and *K* are first turned so as to isolate the system from the culture tube and from the hydrogen supply, and the air is then sucked out of the four-way tube and the wash bottle *J*, after which suction is stopped by turning tap *L* and hydrogen is slowly admitted by turning tap *K* until atmospheric pressure is recorded. Evacuation and refilling with hydrogen are repeated twice. Tap *H* is then turned so as to connect with the culture tube, the hydrogen supply is cut off by means of the tap *K*, the system evacuated and refilled with hydrogen as before. After repeating this process twice it may be taken that any remaining traces of air have been removed and that conditions are now anaerobic. Tap *B* should then be cautiously turned and the intake of hydrogen regulated so that the pellet is pushed along the tube to a point about 12 in. from the tap. This is necessary because gas production is frequently preceded by a certain amount of absorption before the liquid and the gas above it attain equilibrium, and unless sufficient space is allowed the pellet may be drawn through the tap into the culture tube.

When the apparatus has thus been prepared the tap *B* is given a slight turn so as to close all three parts of the capillary tube, *A*, *C* and *D*. The culture tube *F* is then immersed (to a point just above the bottom of the rubber bung) in a thermostat. The end of the capillary tube *D* also dips below the surface of the water in the thermostat, but the long capillary tube *A* extends outside the thermostat and is supported in a horizontal position. Tap *B* is now turned so as to connect *D* and *C* but not *A*. Expansion of the gas in the culture tube and its connexions, due to rise in temperature, therefore finds an outlet through *D* and does not affect the position of the pellet. Moreover, if the conditions are anaerobic, excess hydrogen can escape but the water acts as a seal and no air can filter in through *D*. After half an hour tap *B* is turned so as to connect *C* and *E* and shut off *D*, when the position of the pellet is noted as the starting-point of observations.

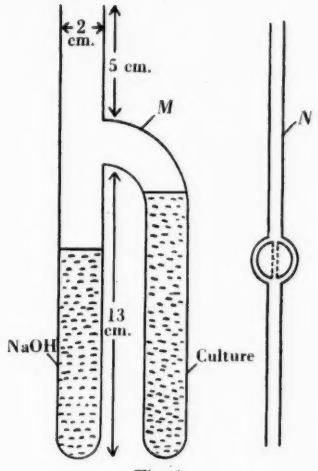

Fig. 4.

Measurement of carbon dioxide by absorption and titration

If the total amount of carbon dioxide evolved by a strain of *Streptococcus* or *Lactobacillus* or by a mixed culture, such as a starter, is required, it must be borne in mind that the capillary tube method gives values only for the gas in excess of that in solution. For measuring the total amount evolved an h tube similar to that shown in Fig. 4 (*M*) was found satisfactory. The dimensions shown may of course be increased so that the tube will hold more culture fluid and therefore evolve a larger quantity of gas. 30 ml. of culture fluid and a suitable inoculum of the test organism are placed in the side arm and 20 ml. of normal sodium hydroxide in the straight arm. A tightly fitting sterile rubber bung is inserted, the seal completed with molten pitch and the tube incubated together with a similar control tube containing no inoculum. At the end of the incubation period the sodium hydroxide is withdrawn by

pipette and titrated in the presence of barium chloride. The difference in titration value between inoculated and control tubes represents the carbon dioxide produced during growth of the organism.

If anaerobic conditions are required, the **h** tube is fitted with a bung carrying a vacuum tap *N*, through which the contents of the tube can be exhausted and replaced by hydrogen by the method previously described, the process being repeated twice to wash out residual traces of air.

REFERENCES

(1) Cranston, J. A. (1930). *Biochem. J.* **24**, 525.
(2) Hassouna & Allen (1939). *J. Dairy Res.* **10**, 7.

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206. GAS PRODUCTION BY *BACTERIUM COLI* AND BY LACTIC ACID STREPTOCOCCI UNDER DIFFERENT CONDITIONS

By M. M. HASSOUNA and L. A. ALLEN
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(With 8 Figures)

In a previous paper (Allen (1)) some methods have been described for measuring gas production by micro-organisms. The objects of the work described here were (1) to measure the gas production (if any) by pure strains of lactic acid streptococci and by starters growing in milk; (2) to measure the total gas evolved by *Bact. coli* in the early stages of growth in different media, particularly milk; (3) to find the effect on gas production by *Bact. coli* of growing *Str. lactis* in the same medium with it and to measure the proportion of *Str. lactis* relative to *Bact. coli* required to inhibit gas production completely—information which has a bearing on the defects due to coliforms growing in the early stages of cheese making; (4) to find the influence on gas production by *Bact. coli*, with and without the concomitant growth of *Str. lactis*, of subjecting it to anaerobic conditions from the outset; and (5) to measure the effect due to the hydrogen alone (by absorbing the carbon dioxide) on the gas produced by *Bact. coli* under different conditions.

The strain of *Bact. coli* used throughout was freshly isolated from cow dung and showed vigorous gas production in the usual sugar broth tubes. The culture of *Str. lactis* was a vigorous strain kindly supplied by Dr J. G. Davis and originally obtained from Prof. Orla Jensen. Both cultures were purified by exhaustive plating before being used.

The media used were either peptone-yeast extract sugar broths or separated milk (raw, heat sterilized or evaporated), and in these carbon dioxide is dissolved to a considerable extent, not only by virtue of its solubility in water but because of its adsorption by the colloidal particles of the medium. The solubility decreases as acidity is developed by the growth of the organisms. Moreover, it was found that, in separated milk at any rate, considerable quantities of hydrogen are absorbed. Added to these observations are the known inherent variability of bacterial cultures and the fact that, in the experiments with the capillary tube method, gas production was observed in the earlier stages of growth only and not when the growth had proceeded to completion. It is not to be expected, therefore, that exact correlation between duplicate experiments will be obtained. The capillary tube method is chiefly of value in showing the extent to which gas formation may result in evolution

of gas outside the medium and in demonstrating major differences in this respect which may result from alteration of the conditions. The graphs of gas production shown in the following pages should be viewed in this light and not regarded as illustrating a mechanism which reproduces results with mathematical precision.

Experiments using the capillary tube method

(1) *Gas production in sterilized separated milk, under initially aerobic conditions*

5 ml. of sterile separated milk were pipetted into each of five culture tubes which were inoculated at the same time and placed together in the thermostat

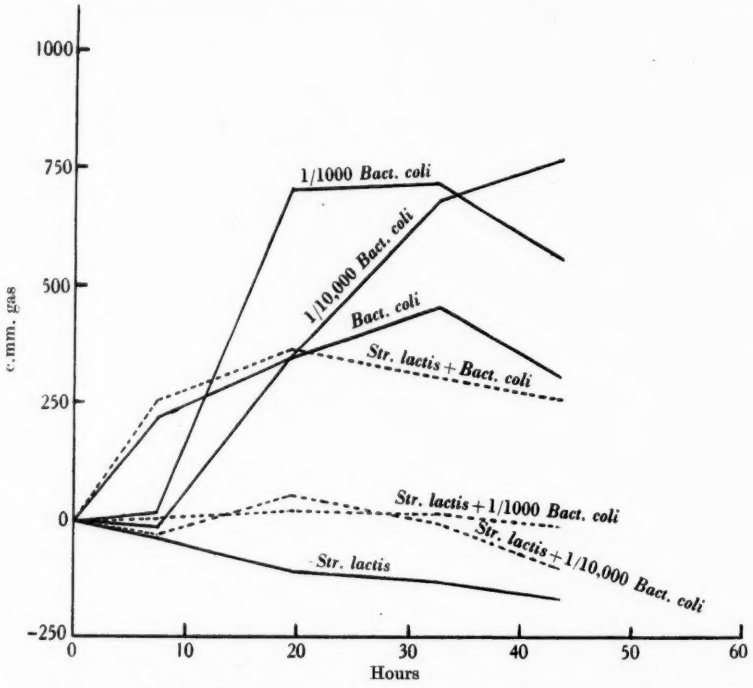

Fig. 1. Gas production in sterilized separated milk by *Bact. coli*, by *Str. lactis* and by mixtures of different proportions of the two organisms. Conditions initially aerobic.

(at a temperature of 26° C.), each tube being connected with a separate capillary tube. The inocula were prepared from a 12 hr. milk culture of *Bact. coli* and a 24 hr. milk culture of *Str. lactis* and were introduced into the culture tubes as follows:

Tube (1) 1 ml. of *Bact. coli* culture.

Tube (2) 1 ml. of 1/10 dilution of *Str. lactis* culture.

Tube (3) 1 ml. of *Bact. coli* culture + 1 ml. of 1/10 dilution of *Str. lactis* culture.

Tube (4) 1 ml. of 1/100 dilution of *Bact. coli* culture.

Tube (5) 1 ml. of 1/100 dilution of *Bact. coli* culture + 1 ml. of 1/10 dilution of *Str. lactis* culture.

The total volume of liquid in each tube was equalized by the addition of sterile saline where necessary.

Gas formation was observed in all tubes except that containing *Str. lactis* alone, where a slow absorption occurred and the mercury pellet was sucked back.

In a second experiment similar observations were made with seven tubes containing a range of different inocula which included 1 ml. of 1/1000 dilution and 1 ml. of 1/10,000 dilution of *Bact. coli*. The results are shown graphically in Fig. 1. The main conclusion to be drawn is that the growth of *Str. lactis* failed to inhibit gas production when the inoculum of *Bact. coli* was as high as 1 ml. or 1/100 ml. but succeeded when the inoculum was reduced to 1/1000 or 1/10,000 ml. The numbers of *Bact. coli* in the milk at the commencement in the two latter cases were respectively 250,000 and 25,000 per ml. The culture of *Str. lactis* was added in approximately the amount used in cheese-making, and the number of organisms present at the commencement of the experiment was 18,000,000 per ml. Counts of both types of bacteria were found by plating in triplicate, the *Bact. coli* on nutrient agar and the *Str. lactis* on yeast extract dextrose milk agar.

It may be noticed that the growth of *Str. lactis* alone results in a slight absorption of gas, and a similar effect was noticed under both aerobic and anaerobic conditions with different organisms in the course of the experiments. It is for this reason that the pellet of mercury must be placed at a reasonable distance from the tap at the commencement of observations.

(2) *Gas production in fresh clean milk under initially aerobic conditions*

The experiments were carried out in a similar way to those just described with the exception that fresh unheated clean milk was used. Two separate series of tests were made, and in both cases gas production was again almost inhibited by *Str. lactis* when the inoculum of *Bact. coli* was 1/1000 ml., but not when it was greater than this. The approximate plate counts of *Str. lactis* and *Bact. coli* respectively at the commencement of the experiment were 61,000,000 and 600,000 per ml. The results of one series of tests are expressed in Fig. 2.

It may be noted that, although when testing gas production by *Bact. coli* alone, a smaller inoculum generally tends to a more marked lag phase, the subsequent rate of gas production bears no relation to the size of the inoculum.

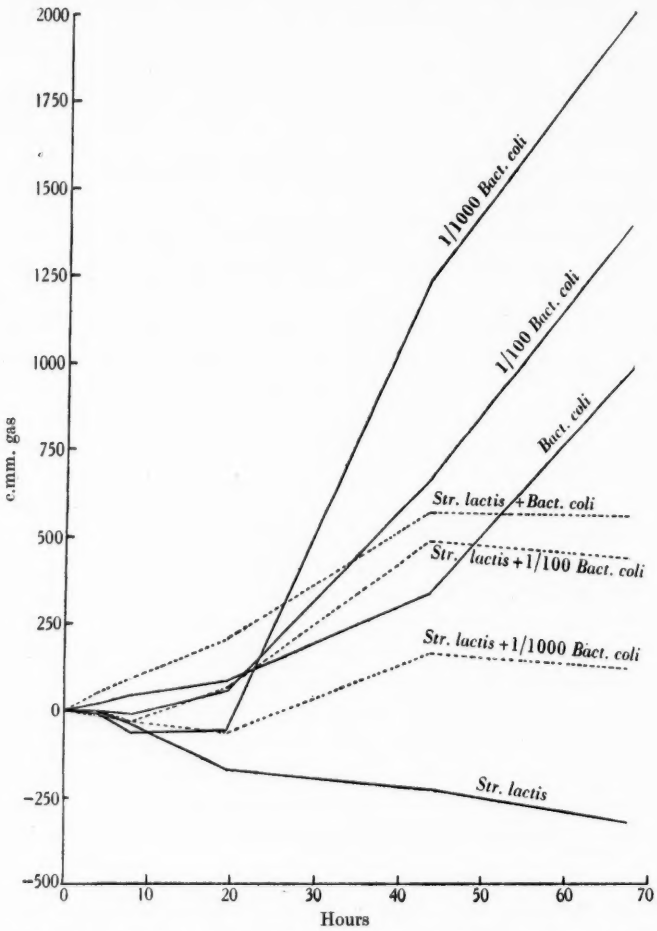

Fig. 2. Gas production in clean raw milk by *Bact. coli*, by *Str. lactis* and by mixtures of different proportions of the two organisms. Conditions initially aerobic.

(3) *Effect on gas evolution from sterilized separated milk of absorbing the carbon dioxide in alkali*

In this series of experiments 5 ml. quantities of sterilized separated milk, contained in one arm of a series of h tubes (the other arm of which contained 10% caustic soda), were inoculated respectively with *Str. lactis*, with *Bact. coli* and with mixtures of these two organisms in different proportions. The conditions were initially aerobic. Fig. 3, illustrating the results, shows that gas evolution was negligible in all tubes. For the first 44 hr. very little movement of the mercury pellet was observed. This was followed by a slight rise,

varying with the different tubes, to be followed in turn by an absorption. After 113 hr. four of the six tubes showed a negative pressure (the mercury pellet having been drawn nearer the tap) and the remaining two tubes a very slight positive pressure. One can only conclude from this that the gas evolved by *Bact. coli* growing in separated milk consists of carbon dioxide only, and when this is absorbed by alkali the effect due to hydrogen is negligible.

In order to confirm this observation a second series of experiments was carried out, using seven culture tubes, containing sterile separated milk and inoculated at the same time as follows:

(1) Duplicate straight tubes, inoculated with 1 ml. of 1/100 dilution of a 12 hr. milk culture of *Bact. coli*, to show total gas evolution (CO_2+H_2).

(2) Duplicate h tubes (containing alkali in one arm), inoculated as in (1), to show the effect due to hydrogen alone.

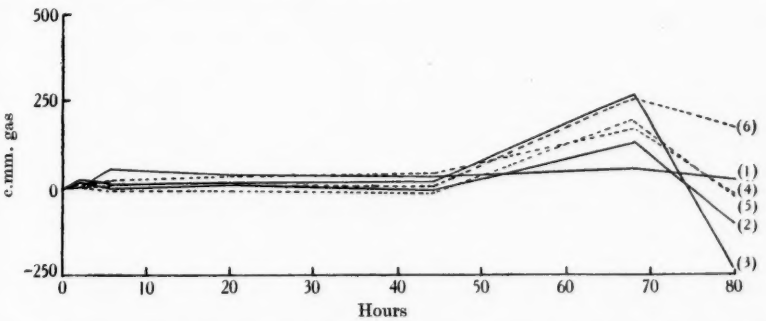

Fig. 3. Gas production in h tubes (the CO_2 being absorbed by alkali) in sterilized separated milk by *Str. lactis*, by *Bact. coli* and by mixtures of different proportions of the two organisms. Conditions initially aerobic. The inocula were as follows: (1) *Bact. coli*, (2) *Str. lactis*, (3) 1/1000 *Bact. coli*, (4) *Str. lactis* + *Bact. coli*, (5) *Str. lactis* + 1/100 *Bact. coli*, (6) *Str. lactis* + 1/1000 *Bact. coli*.

(3) Duplicate h tubes inoculated with 1 ml. of 1/100 dilution of a 12 hr. milk culture of *Bact. coli* + 1 ml. of a 1/10 dilution of a 24 hr. culture of *Str. lactis*.

(4) One straight tube, inoculated as in (3) with a mixture of *Str. lactis* and *Bact. coli*.

Fig. 4 shows that total gas production by *Bact. coli* alone is considerable, in the presence of *Str. lactis* it is much less, whereas in the tubes in which carbon dioxide was absorbed by alkali, *Bact. coli* (either alone or in combination with *Str. lactis*) evolves a negligible amount of gas after 20 hr., following which there is an appreciable absorption.

In a third experiment the gas production by *Bact. coli* in sterile separated milk was tested under conditions which were strictly anaerobic from the commencement. Six tubes were inoculated simultaneously with 1 ml. of

1/100 dilution of a 12 hr. milk culture of *Bact. coli*, three tubes being straight while three were h tubes containing alkali to absorb carbon dioxide. The system in each case was evacuated and filled with hydrogen as described in the previous paper(1). Fig. 5 shows that the total gas production was appreciable in each of the first three tubes, although there were marked individual

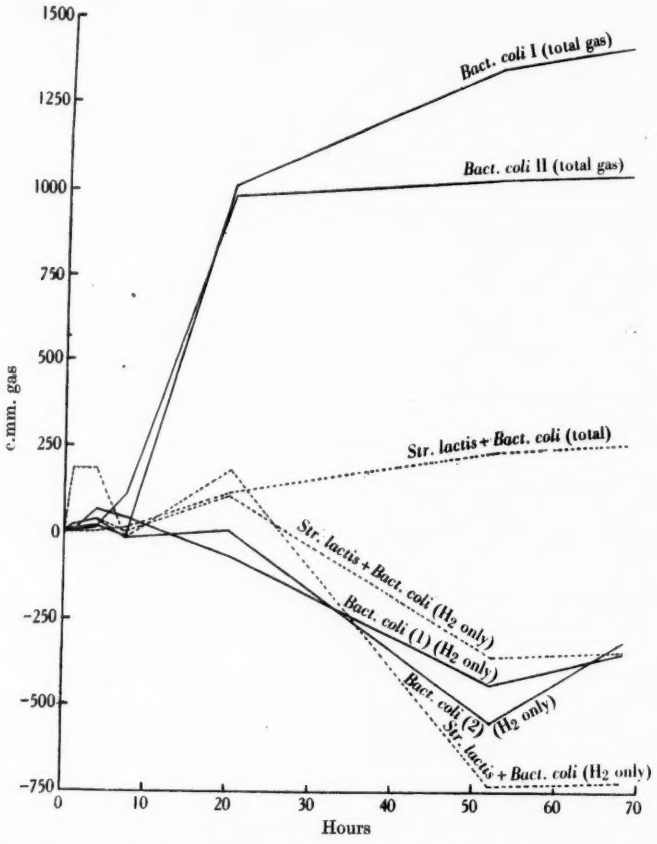

Fig. 4. Gas production in sterilized separated milk by *Bact. coli*, by *Str. lactis* and by a mixture of the two organisms. Showing comparison between total gas production and gas production in h tubes when CO_2 is absorbed by alkali. Conditions initially aerobic.

differences. In the h tubes, however, no gas evolution was observed and in one tube an absorption occurred in the later stages of the experiment. It is evident, therefore, that whether the conditions are initially aerobic or anaerobic the pressure due to gas evolved by *Bact. coli* growing in milk is due to carbon dioxide only, the hydrogen being absorbed in some way by the constituents of the medium.

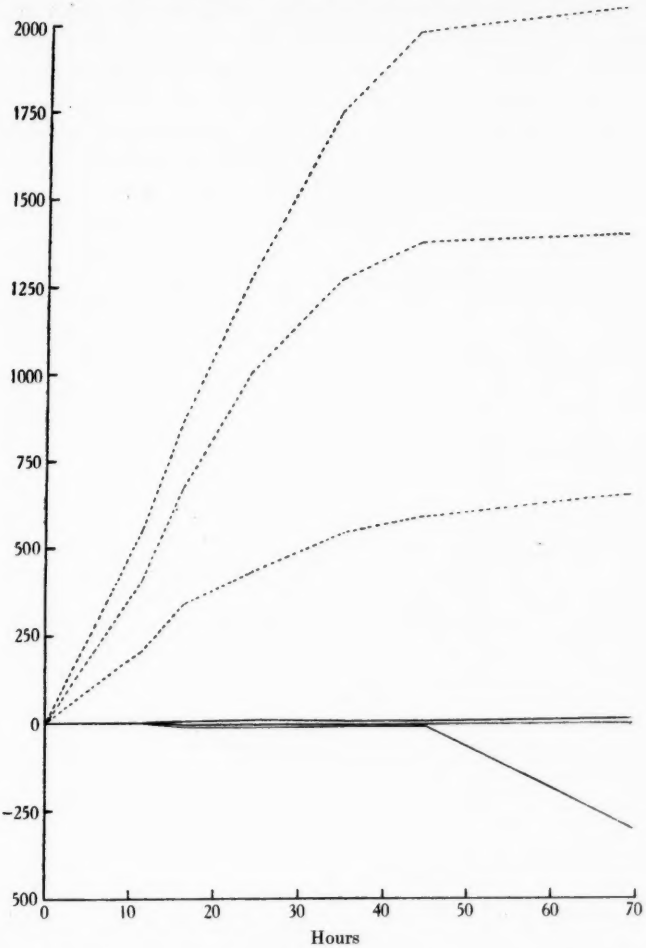

Fig. 5. Gas production in sterilized separated milk by *Bact. coli*. Conditions aerobic. (*a*) Total gas production (broken line). (*b*) Gas production in h tubes, in which the CO_2 is absorbed by alkali (continuous line).

(4) *Gas evolution from lactose and dextrose broths by* Bact. coli

Since the gas produced by the carbohydrate metabolism of *Bact. coli* is known to result in equal volumes of hydrogen and carbon dioxide, it was of interest to see whether the absorption of hydrogen under the conditions tested was peculiar to a milk medium or whether it occurred appreciably in sugar broths.

Lactose broth (containing 2% lactose, 1% peptone, 0·3% marmite, 0·25% sodium chloride and sterilized by autoclaving at 250° F. for 20 min.)

was pipetted into each of six culture tubes (three were straight tubes while three were h tubes containing alkali) which were each inoculated at the same time with 1 ml. of a thin saline suspension of a 12 hr. culture of *Bact. coli* on nutrient agar. Conditions were strictly anaerobic from the commencement. Fig. 6 shows the results of gas evolution, and it is evident that considerable

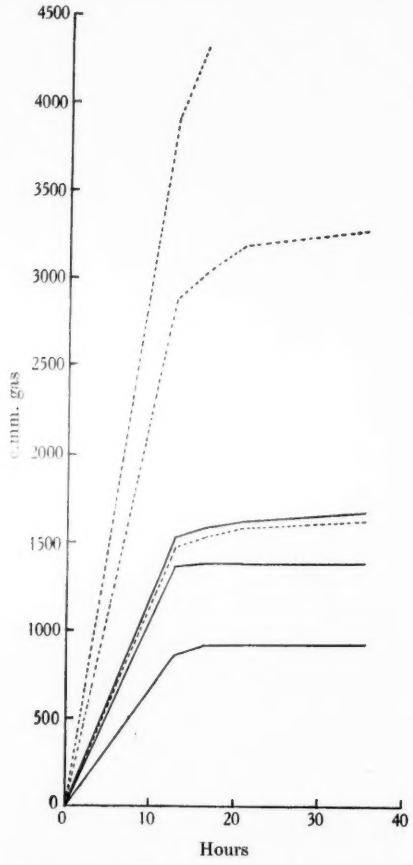

Fig. 6. Gas production in lactose broth by *Bact. coli*. Conditions anaerobic. (*a*) Total gas production (broken lines). (*b*) Gas production in h tubes, where CO_2 is absorbed by alkali (continuous lines).

gas was evolved from both the straight tubes and from those containing alkali, although on an average there was more than twice as much evolved in the former case.

Similar experiments under initially aerobic conditions were made with lactose and dextrose broths. One straight culture tube and two h tubes containing dextrose broth and one h tube containing lactose broth were

inoculated with *Bact. coli* as in the previous experiment. Evolution of gas was observed in all tubes, as shown in Fig. 7, where it may be seen that the total gas evolved from dextrose was nearly twice the amount of hydrogen alone.

Finally six tubes (three containing sterilized separated milk and three containing lactose broth) were sown with equal inocula from the same culture of *Bact. coli* (1 ml. of a saline suspension of a 24 hr. slope culture on nutrient agar). One tube of each medium was an ordinary straight tube for showing

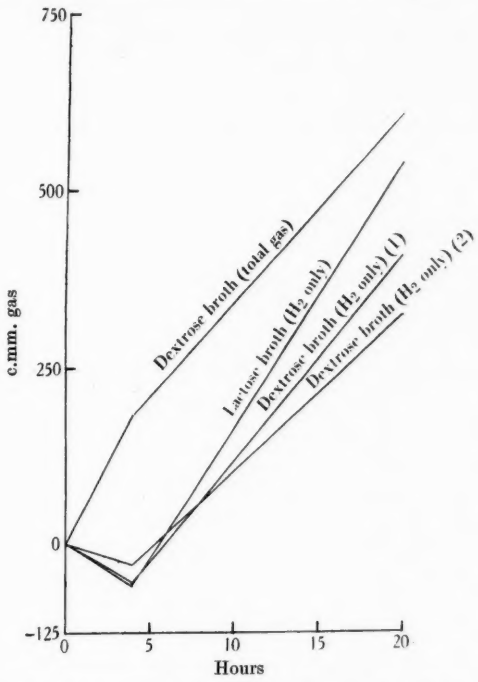

Fig. 7. Gas production in dextrose and lactose broths. Conditions initially aerobic. Comparison between total gas produced in dextrose broth and gas produced in both media in h tubes when CO_2 is absorbed by alkali.

total gas produced, while the remaining two were h tubes containing alkali to absorb carbon dioxide. Conditions were anaerobic. The results are shown in Fig. 8. It is apparent that, in the milk medium, no gas pressure is shown when the carbon dioxide is absorbed, although there is considerable total gas evolved from the straight tube. With lactose broth there is abundant gas production in both straight tube and h tubes, the former yielding higher quantities than the latter.

It appears from these experiments that the complete absorption of hydrogen during the growth of *Bact. coli* is a peculiarity of the milk medium and

does not apply to sugar broths. It may be mentioned that, in every case where no gas was detected, the growth of the inoculated *Bact. coli* was sufficient to curdle the milk.

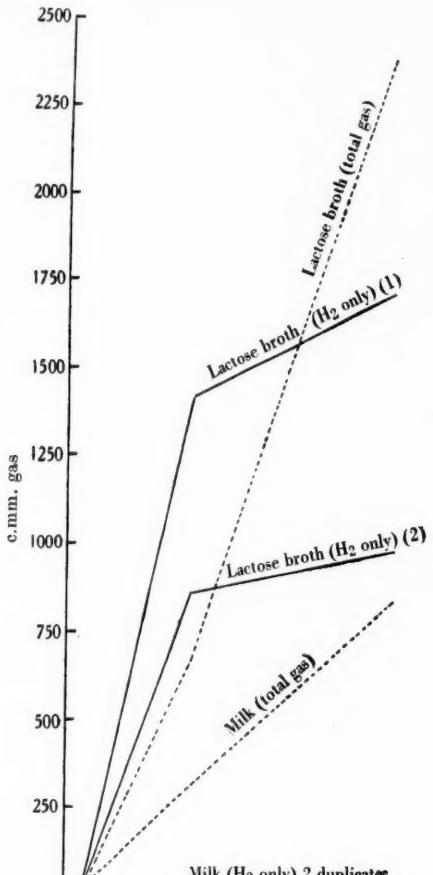

Fig. 8. Gas production by *Bact. coli* in sterilized separated milk and in lactose broth. Showing comparison between total gas and gas produced in h tubes, where CO is absorbed by alkali. Conditions anaerobic.

Gas production by lactic acid bacteria

The group of lactic acid bacteria which form almost entirely lactic acid from carbohydrates generally produces negligible quantities of gas, while, on the other hand, those which give rise to appreciable amounts of metabolic products other than lactic acid (Orla Jensen's *Betacocci* or *Betabacteria*) may form considerable gas. Observations recorded in the literature from time to time tend to the conclusion that gas production by these species is subject to considerable variation according to the conditions of culture and is not the constant property which is associated, for instance, with coliform bacteria.

It was decided to test the gas-producing power, under different conditions, of a variety of lactic acid streptococci which had been kept subcultured for some time in different media. Gas evolved outside the medium and therefore capable of exerting pressure was detected by the capillary tube method. Total carbon dioxide, including that normally dissolved in the medium, was estimated by titration, using large h tubes containing alkali as described previously (1).

In the first series of experiments strains of *Str. lactis*, *Str. cremoris* and *Str. paracitrovorus*, which had been cultivated in a variety of media including litmus milk, dextrose yeast extract litmus milk, tryptic casein digest agar and evaporated milk, were tested for gas evolution by the capillary tube method in separated milk, yeast extract separated milk and evaporated milk. No evidence of evolved gas was recorded in duplicate experiments with any of these organisms. Mixed cultures of the same three strains also gave a negative result when tested in separated milk. When the carbon dioxide formed in separated milk was estimated by titration in h tubes it was found that both *Str. lactis* and *Str. paracitrovorus* produced small but definite amounts, indicating that the gas produced by these organisms is capable of being dissolved by the medium and thus exerts no external pressure.

Similar tests with two strains of *Str. diaceti lactici* and one of *Leuconostoc mesenteroides* produced more positive results. One strain of the former organism showed appreciable gas formation in separated milk while the other strain had apparently lost its ability to do so. *L. mesenteroides* gave a negative result when tested by the capillary tube method in separated milk but formed appreciable gas in milk containing 1 % dextrose and 0·3 % yeast extract. The experiments were carried out in duplicate with consistent results. It was found, however, that the gas-producing strain of *Str. diaceti lactici* when kept subcultured for several months in Robertson's cooked meat had lost its ability to form gas and the carbon dioxide estimated by the titration method was then negligible in amount.

Since it has been reported by Hammer (2) that a strain of *Str. paracitrovorus* was found by him to be capable of forming gas in a tin of evaporated milk in sufficient quantity to bulge the tin, although it formed negligible amounts in tubes of sugar broths, it was decided to test the strain of *Str. paracitrovorus*

used in the work just described. For this purpose six tins of evaporated milk were first incubated for 4 days at 30° C. None showed any change in its appearance. Five tins were then punctured with aseptic precautions, three were inoculated with *Str. paracitrovorus*, and all five tins resealed. The six tins were then reincubated at 30° C. After 4 days' incubation the three inoculated tins showed definite bulging at both ends while neither of the two tins which had been opened but not inoculated, nor the tin which had not been opened, showed any change in appearance.

It was surprising to find that the same organism which had not shown gas formation in evaporated milk in the capillary tube method should yet do so when inoculated into a tin of evaporated milk. Its peculiarity in this respect was also shown in one flask of sterile separated milk which had been inoculated with the organism, closed with a tightly fitting rubber bung, and incubated at 20° C. After several weeks gas formation was observed and at one period was sufficient to blow out the bung. No evidence of contamination by other organisms could be obtained.

Conclusions

1. The capillary tube method is convenient for measuring approximately the volume of gas (other than that dissolved in the medium) evolved during bacterial growth under conditions which are initially either aerobic or anaerobic.

2. With vigorous strains of *Str. lactis* and of *Bact. coli* growing together in separated milk it was found that when the inocula were respectively 1/10 and 1/1000 ml. (of a young milk culture in both cases) gas production by the latter organism was inhibited by the growth of the former. With a larger inoculum of *Bact. coli* gas formation was not prevented, though the extent to which it was reduced depended on the relative sizes of the inocula of the two organisms.

3. In sterile separated milk the gas evolved during the growth of *Bact. coli* was found to be carbon dioxide only and when this was absorbed by alkali no gas evolution could be detected. In dextrose or lactose broths under the same conditions, however, both hydrogen and carbon dioxide were formed in accordance with the usual conceptions of the carbohydrate metabolism of coliform organisms. It appears, therefore, that both hydrogen and carbon dioxide are formed from the lactose in milk but that the hydrogen is used in some secondary reaction with the constituents of the medium and does not appear as gas. Contrary to the usual belief that gaseous defects due to the growth of coliform organisms in milk and milk products are due more to the insoluble hydrogen than to the soluble carbon dioxide, it would appear that carbon dioxide is probably the only gas concerned and that the effect of the hydrogen is negligible.

4. Lactic acid streptococci of the type commonly used in starters (e.g. *Str. lactis*, *Str. cremoris*, *Str. paracitrovorus*), whether in pure or in mixed cultures, gave no evidence of gas formation by the capillary tube method in

either separated milk, separated milk containing yeast extract, or in evaporated milk. Small amounts could be detected by the titration method, using large h tubes in which carbon dioxide was absorbed by alkali during growth. The small amounts formed were therefore easily soluble in the medium and produced no external evolution of gas.

One strain of *Str. diaceti lactici* showed considerable evolution of gas from separated milk, while a strain of *Leuconostoc mesenteroides*, though not evolving gas from separated milk, produced considerable quantities from milk containing dextrose and yeast extract.

5. There is a capricious quality noticeable in the gas formation by lactic acid streptococci. Thus *Str. paracitrovorus*, when tested in either separated milk or evaporated milk by the capillary tube method, showed no gas formation but when inoculated into tins of evaporated milk it produced sufficient gas to bulge the ends of the tins. The same strain on one occasion produced sufficient gas in a flask of separated milk to blow out the bung.

REFERENCES

(1) ALLEN, L. A. (1939). *J. Dairy Res.* **10**, 1.
(2) HAMMER, B. W. (1927). *Iowa St. Coll. J. Sci.* **2**, 1.

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207. PROTEIN METABOLISM AND ACID PRODUCTION BY THE LACTIC ACID BACTERIA IN MILK. INFLUENCE OF YEAST EXTRACT AND CHALK

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Though the lactic acid bacteria are recognized primarily as saccharolytic, several workers have recorded observations on their slow proteolytic activity. Von Freudenreich (1) was the first to record the fact that cultures of these organisms in milk, to which chalk had been added to neutralize the acidity, formed appreciable amounts of soluble nitrogen, and these findings were confirmed by Orla-Jensen (2), Barthel (3), and Barthel & Sandberg (4). Anderegg & Hammer (5), in a study of a large number of strains of *Str. lactis*, found an increase in soluble nitrogen in some cases and a decrease in others, while occasionally the same strain differed in different tests. In general, cultures which clotted rapidly were more inclined to proteolysis than those which were slower in forming acid. *Str. citrovorus* and *Str. paracitrovorus* did not cause protein breakdown. Addition of 0·3 % peptone to the milk tended to retard proteolysis or to increase negative values while addition of chalk resulted in more extensive proteolysis. Barthel & Sadler (6) found that starters consisting of mixed cultures of streptococci produced more extensive proteolysis than single species, indicating a symbiotic effect. Sherwood & Whitehead (7) tested the proteolytic powers of several strains of *Str. cremoris* in chalk milk cultures and found some active and some comparatively inactive. Two strains appear to have formed surprisingly large amounts of non-protein nitrogen. In general they found that acid-producing power was linked with proteolytic power.

Lactobacilli are credited by some workers with more pronounced proteolytic effects than the streptococci and for this reason are regarded as important in the later stages of cheese ripening. Kulp & Rettger (8) found that *Lactobacillus acidophilus* and *L. bulgaricus* in milk cultures formed amino acids as well as proteoses and peptones. Since these authors also found that 3 % lactic acid, added to sterile milk, resulted after incubation in approximately the same amount of proteoses and peptones as the milk inoculated with the lactobacilli they concluded that the first stage in proteolysis by these organisms is induced by the hydrolytic action of the acid they form. On the other hand, Sherwood (9) regards the lactobacilli as less able to attack protein than the streptococci though they may be capable of breaking down proteose and peptone to subpeptone.

Factors influencing the fermenting power of the lactic acid bacteria have also been studied by a number of workers. Orla-Jensen (10) originally showed

the importance of the nitrogen source, and the same author (11) has indicated the influence of *p*H and of certain activating substances. Orla-Jensen & Jacobsen (12) showed that the acid production of some species of lactic acid bacteria in milk is increased by addition of 0·0025 % yeast extract.

The nitrogen source is probably a very important factor controlling the effect of lactic acid bacteria in cheese ripening. Thus their greater activity in cheese made from market milk compared with that in cheese made from milk of a very low bacterial count, and the more extensive proteolysis which occurs in the former cheese, are thought to be due to the influence of the proteolytic bacteria in the market milk in providing protein degradation products for the starter organisms in the early stages (Allen (13)). Many of the lactic acid bacteria isolated during the course of cheese ripening are of the type which is unable to form acid rapidly in milk unless an extra nitrogen source, such as yeast extract, is added, and it is safe to assume that their influence in cheese ripening will be enhanced if such a source is present.

A survey of the literature indicates that the effect of the lactic acid bacteria on the proteins of milk and cheese has not been by any means exhaustively studied, results tend to be inconclusive, nor is the influence of various conditions on the process understood. Amongst the reasons responsible for this, two may be mentioned. (1) The well-known variability of this group may result in different strains of the same organism possessing very different proteolytic powers. This would apply particularly, perhaps, to the difference between cultures freshly isolated from a natural source and those which had been kept in artificial culture for some time. (2) Methods of demonstrating proteolysis may have suffered from the lack of suitable protein precipitants which would effect a reliable division between, say, proteins, proteoses and peptones, and amino acids.

For these reasons it was decided to make a comprehensive study of the acid production, viability and effect on the protein in milk of a wide range of streptococci and lactobacilli, and to investigate the effect on these properties of adding yeast extract or chalk or both substances to the medium. This decision was encouraged by the fact that recently Rowland (14) has devised more consistent methods of obtaining fractions of protein degradation products in milk by the use of suitable precipitants.

Experimental

Narrow-necked sample bottles with a capacity of about 600 ml., containing 490 ml. of separated milk, were sterilized by intermittent steaming. For experiments with milk containing yeast extract 10 ml. of water containing 1·5 g. marmite were sterilized in the autoclave and added to each bottle under aseptic conditions, so that the final concentration of yeast extract was 0·3 %. To bottles not containing yeast extract 10 ml. of sterile water were added so that the concentration of milk constituents was the same in each series. When chalk was to be used 20 g. of finely divided chalk were first added to each

bottle and sterilization effected in the hot-air oven. Milk was then added to the bottles, sterilized by steaming, and yeast extract or water added as before. It is to be noted that the milk and yeast extract were never sterilized together, so that there was no question of activating substances being produced by reaction between the two materials under the influence of heat. Comparative experiments with and without yeast extract for the same organism were always made with milk from the same original bulk.

The inoculum consisted of 10 ml. of a 1/10 dilution of a 24 hr. milk culture of the organism, and the bottles were then fitted with sterile rubber bungs and incubated at 20° C. Samples were abstracted by means of a pipette under aseptic conditions at appropriate intervals. Separate bottles were used for obtaining counts and for chemical data respectively, and all determinations were made in duplicate. In the earlier experiments, in order to minimize risk of contamination through opening bottles for the abstraction of samples, chemical determinations were made by using a series of large test-tubes for each experiment, all containing the same milk and inoculated at the same time. The whole contents of one tube (about 45 ml.) were then used at each stage. It was found occasionally, however, that discordant results were obtained owing to the differences in growth of the bacteria in individual tubes. For this reason it was decided to use large bottles as described, and it was found that the narrowness of the necks, combined with strictly aseptic precautions, obviated contamination over quite long periods.

Chemical methods

Cultures to which no chalk had been added were thoroughly shaken before each test for a considerable time to break up the coagulum and reduce it to a viscous homogeneous fluid. One hour was then allowed to elapse in order to permit occluded air bubbles to rise to the surface before abstracting the sample for analysis. Cultures containing chalk were shaken thoroughly at least once, and frequently twice, a day during the period of incubation in order to neutralize the developed acidity, the bung being loosened under aseptic conditions to permit the escape of carbon dioxide formed by the action of the acid on the chalk. Prior to each test the bottle was shaken, allowed to stand for 1 hr. for the chalk to settle and samples for analysis pipetted from the supernatant fluid. This was found to be quite satisfactory because no coagulation occurred and the culture remained liquid.

Titratable acidity. 10 ml. of culture were diluted with 10 ml. of distilled water in a porcelain basin, a few drops of phenolphthalein added and the mixture titrated with *N*/10 barium hydroxide to a faint permanent pink colour.

Total alcohol-formalin titration. To the neutralized culture after determination of the acidity a mixture (previously neutralized to phenolphthalein) of 80 ml. of absolute alcohol, 5 ml. of 40 % formaldehyde, 10 ml. of water and 1 ml. of 25 % sodium chloride solution were added and the titration with

barium hydroxide continued. The second titration then gave a measure of the total amino nitrogen in the culture.

Alcohol-formalin titration of alcohol-soluble portion. 10 ml. of culture were transferred to a 100 ml. graduated flask, diluted with 10 ml. of water and neutralized to phenolphthalein with normal sodium hydroxide. Neutralized absolute alcohol was then added to the 100 ml. mark, the mixture shaken thoroughly and allowed to stand for 1 hr., after which it was filtered through a dry filter paper. After washing the precipitate with a small quantity of neutral 80% alcohol, 5 ml. of 40% formaldehyde were added to the filtrate and the latter was titrated with barium hydroxide. A blank titration with 5 ml. of formaldehyde and 20 ml. of distilled water was subtracted from this reading. The final figure was a measure of the amino nitrogen in the lowest protein decomposition products, soluble in 80% alcohol.

Nitrogen distribution. The methods evolved by Rowland (14) for precipitation of the proteins in milk were applied, using the semi-micro procedure. Determination of the total nitrogen by the modified Kjeldahl method in bacterial cultures in sterilized milk gives figures which are, of course, comparable in every way with those obtained by this author for fresh milk. When using the acetic acid-sodium acetate precipitant, however, certain differences in the nature of the precipitates obtained from fresh milk and in that from a culture in sterilized milk must be borne in mind. Thus Rowland found that the maximum precipitation of casein in fresh milk is obtained by adding 1 ml. of 10% acetic acid and 1 ml. of normal sodium acetate solution to 10 ml. of milk suitably diluted, and that the filtrate from this mixture contains albumin, globulin, proteose, peptone, amino acids, etc. It was found, on the other hand (Rowland (15)), that denaturation of albumin and globulin took place rapidly in milk heated at 75° C. and above, and was complete, for instance, in 5–10 min. at 100° C. In such milk the acetic acid-sodium acetate mixture referred to precipitated casein, albumin and globulin, and since, in the experiments described in this paper, the milk used for cultures was sterilized by intermittent steaming (30 min. on three successive days) similar considerations apply. Furthermore, the growth of bacteria in such milk will result in the formation of bacterial protein, some of which may be precipitable by acetic acid. The reactions of bacterial proteins have not been extensively studied, but Thomas (16), for instance, isolated two proteins from yeast, one of which somewhat resembled casein in its reactions and was precipitated by acetic acid. It follows, therefore, that the precipitate formed by adding the acetic acid-sodium acetate mixture to the milk cultures used in these experiments will consist of casein, albumin, globulin, yeast protein (in the case of cultures to which yeast extract has been added) and such synthesized bacterial proteins as are precipitated by acetic acid. It will, in fact, contain nearly all the true proteins of the culture, while the filtrate will contain the proteose-peptone fraction plus the lower decomposition products such as amino acids and ammonium salts.

In view of these considerations the methods adopted were as follows:

Total nitrogen was estimated by digestion of 10 ml. of a 1/10 dilution of the culture with 5 ml. of H_2SO_4, 2 g. of K_2SO_4, 0·2 g. of $CuSO_4$, and 2 drops of selenium oxychloride in a Kjeldahl flask, the contents then being made alkaline, steam distilled into $N/70$ H_2SO_4, and the excess acid titrated with $N/70$ sodium hydroxide.

Protein nitrogen. 10 ml. of the milk culture were diluted, treated with acetic acid and sodium acetate as recommended by Rowland (15), and the nitrogen content of the filtrate estimated by the method outlined above. The difference between this figure and that for the total nitrogen represented the nitrogen present as protein.

Non-protein nitrogen. 10 ml. of culture were treated with 15 % trichloracetic acid solution to give a final concentration of 12·5 % as recommended by Rowland (15) and the nitrogen content of 10 ml. of the filtrate determined.

Proteose-peptone nitrogen was obtained by subtracting the value for non-protein nitrogen from that for the nitrogen in the filtrate from the acetic acid-sodium acetate precipitation.

Bacteriological methods

The strains of streptococci and lactobacilli used were purified by plating several times on yeast extract-dextrose-milk agar and typed by observing their reactions in carbohydrate broths and in litmus milk, yeast extract-litmus milk, dextrose-litmus milk and yeast extract-dextrose-litmus milk. Most of the lactobacilli had been previously isolated from various silages or from dairy products, and an account of their characters is given by Allen & Harrison (17).

Counts of the bacteria in the bottle cultures were obtained by abstracting 10 ml., diluting with 90 ml. of saline, preparing further dilutions in tubes of saline and inoculating duplicate quantities of 1 ml. from these into tubes of yeast extract-dextrose-litmus milk, observing the highest dilution in which acid was formed after incubation. Where duplicates differed the higher value was recorded.

(1) *Experiments with streptococci.*

Six strains of lactic streptococci—*Str. lactis* I and *Str. cremoris* I (both originally obtained from Prof. Orla Jensen), *Str. lactis* II (isolated from raw milk), *Str. cremoris* II (isolated from commercial starter), *Str. paracitrovorus* (originally obtained from Prof. Hammer) and *Leuconostoc mesenteroides* (originally obtained from Prof. Kluyver). The characters of these organisms are shown briefly in Table I.

Separate experiments were conducted with each of these organisms and also with a mixture of the first five streptococci in symbiosis. The figures for total count, acidity, alcohol-formalin titration of the whole culture and of the alcohol-soluble portion, and for the nitrogen distribution (protein, proteose-

Table I. *Characters of streptococci*

Culture	Litmus milk media*				Carbohydrate broths												
	Litmus milk	Yeast extract litmus milk	Dextrose litmus milk	Yeast extract dextrose litmus milk	Arabinose	Xylose	Dextrose	Laevulose	Galactose	Mannite	Lactose	Maltose	Sucrose	Raffinose	Salicin	Dextrin	Inulin	Starch
Str. lactis I	+ + +	+ + +	+ + +	+ + +	−	+	+	+	+	−	+	+	−	−	+	+	−	−
Str. lactis II	+ + +	+ + +	+ + +	+ + +	−	+	+	.	+	−	+	+	−	−	+	+	−	−
Str. cremoris I	+ +	+ +	+ + +	+ + +	−	−	+	+	−	−	+	−	−	−	−	−	−	−
Str. cremoris II	+ + +	+ + +	+ + +	+ + +	−	−	+	.	−	−	+	−	−	−	−	−	−	−
Str. paracitrovorus	±	+	+	+ +	+	−	+	+	+	−	−	+	+	−	−	−	−	−
Leuc. mesenteroides	±	+	+	+ +	−	+	+	+	−	−	+	−	+	−	+	−	−	−

Table II. *Characters of lactobacilli*

Culture	Litmus milk media*				Carbohydrate broths						
	Litmus milk	Yeast extract litmus milk	Dextrose litmus milk	Yeast extract dextrose litmus milk	Arabinose	Xylose	Dextrose	Lactose	Sucrose	Maltose	Salicin
L. plantarum I	+ +	+ + +	+ +	+ + +	+	+	+	+	+	+	+
L. plantarum II	+	+ +	+	+ + +	+	+	+	+	+	+	+
L. plantarum III	+	+ + +	+	+ + +	+	+	+	+	−	+	−
L. casei I	+ + +	+ + +	+ + +	+ + +	+	+	+	+	+	+	+
L. casei II	+ + +	+ + +	+ + +	+ + +	+	−	+	+	−	+	+
L. bulgaricus	+ +	+ + +	+ +	+ + +	−	−	+	+	−	+	−
L. acidophilus	+ + +	+ + +	+ + +	+ + +	−	−	+	+	+	−	−

* The number of plus signs indicate the relative rapidity with which acid was formed in the litmus milk media.

peptone and amino acids, etc.), are given in detail for *Str. lactis* II in Table III. Typical examples of bacterial counts are shown in Tables IV and V and of alcohol-formalin titrations in Table X. A summary of figures for acid development is given in Table VIII and for nitrogen distribution in Table XIII. Each figure shown is the average of duplicates.

There are certain conclusions, following from these results, which are of general application:

(1) The maximum numbers of bacteria in any one experiment are approximately the same whether the culture contains yeast extract or not and this holds good for cultures with and without chalk.

(2) The lactic acid developed by the cultures is greater in milk containing yeast extract than in milk alone. With strains of *Str. lactis* and *Str. cremoris*, which curdle plain milk rapidly, the difference is not so marked, but with *Str. paracitrovorus* and *Leuconostoc mesenteroides*, which form acid very slowly in plain milk, the addition of yeast extract has a relatively large influence. It may be seen that acid continues to be produced long after the cultures are dead.

(3) After the maximum has been attained bacterial numbers decrease more rapidly in the cultures containing yeast extract than in plain milk. In

Table III. *Streptococcus lactis* II

Bacterial counts per ml.

Age of culture		Days 0	1	2	3	4	5	7	15	Weeks 3	4	5	6	8	12
Without chalk	Y.E.*	10^7	10^9	10^9	10^7	10^6	10^5	10^4	10	—	—	—	—	—	—
	No Y.E.	10^7	10^8	10^8	10^8	10^8	10^8	10^7	10^6	10^5	10^4	10	—	—	—
With chalk	Y.E.	10^6	10^8	10^9	—	10^{10}	10^{10}	10^{10}	10^{10}	—	10^{10}	—	—	10^9	10^8
	No Y.E.	10^6	10^8	10^9	—	10^9	10^{10}	10^{10}	10^{10}	—	10^{10}	—	—	10^{10}	10^9

Age of culture (weeks)	Developed acidity ml. $N/10$ per 100 ml. Without chalk Y.E.	No Y.E.	Alcohol-formalin titrations (ml. $N/10$ per 100 ml.) Without chalk: Whole culture Y.E.	No Y.E.	Alc. filtrate Y.E.	No Y.E.	With chalk: Whole culture Y.E.	No Y.E.	Alc. filtrate Y.E.	No Y.E.
0	0·0	0·0	40·8	31·8	15·3	9·6	32·6	25·6	13·4	8·6
2	76·0	73·6	30·8	24·9	13·5	9·8	13·9	11·4	13·7	7·5
4	79·7	77·0	30·6	27·2	13·5	11·1	11·4	8·2	14·4	10·2
6	80·1	71·0	24·7	19·8	13·2	7·5	20·4	11·3	14·1	10·0
10	82·6	72·0	27·6	26·3	15·8	10·8	24·9	14·5	14·9	9·2

Nitrogen distribution (mg. N per 100 ml. culture)

Age of culture (weeks)	Protein Y.E.	No Y.E.	Proteose-peptone Y.E.	No Y.E.	Amino acids, etc. Y.E.	No Y.E.
			Without chalk			
0	530·9	533·0	40·4	37·8	60·7	42·2
6	550·2	546·8	24·2	26·5	57·6	39·7
Difference	+19·3	+13·8	−16·2	−11·3	−3·1	−2·5
			With chalk			
0	405·4	401·9	17·9	18·4	48·0	28·5
12	374·6	377·5	51·9	40·0	44·7	31·3
Difference	−30·8	−24·4	+34·0	+21·6	−3·3	+2·8

* Y.E. in this and in the following tables = yeast extract.

Table IV. *Streptococcus cremoris* I

Bacterial counts per ml.

Age of culture		Days 0	2	4	7	12	15	Weeks 3	4	5	8	12
Without chalk	Y.E.	10^6	10^9	10^8	10^7	10^2	10	—	—	—	—	—
	No Y.E.	10^5	10^8	10^8	10^8	10^7	10^6	10^4	10^2	—	—	—
With chalk	Y.E.	10^5	10^7	10^8	10^8	—	10^8	—	10^9	—	10^{10}	10^9
	No Y.E.	10^5	10^7	10^8	10^8	—	10^9	—	10^9	—	10^9	10^9

Table V. *Streptococcus paracitrovorus*

Bacterial counts per ml.

Age of culture		Days 0	1	2	4	7	12	15	Weeks 4	6	8	10	12	14
Without chalk	Y.E.	10^6	10^6	10^8	10^8	10^9	10^9	10^9	10^8	10^8	10^7	10^5	—	10^4
	No Y.E.	10^6	10^6	10^8	10^8	10^8	10^9	10^9	10^9	10^8	10^8	10^6	—	10^5
With chalk	Y.E.	10^5	10^6	10^8	10^8	10^9	—	10^9	10^9	—	10^8	—	10^7	—
	No Y.E.	10^5	10^6	10^8	10^8	10^8	—	10^9	10^8	—	10^8	—	10^8	—

other words, addition of yeast extract decreases the viability. This effect is very marked in the cultures of *Str. lactis* and *Str. cremoris*, although the amount of extra acid in the yeast extract cultures is not very great. On the other hand, the viability of cultures of *Str. paracitrovorus* and *Leuconostoc mesenteroides* is not affected very much by the presence of yeast extract, though the increase in acid is relatively large. It may be seen, however, that lactic acid is developed much more slowly in the two latter cultures, even in the presence of yeast extract, so that acidity is probably the main factor controlling viability. The viability of all cultures is very much increased by the addition of chalk because of its acid-neutralizing properties, and it is, perhaps, significant that the detrimental influence of yeast extract is not apparent in such cultures.

(4) With the exception of the two strains of *Str. cremoris*, all cultures of single species show an appreciable increase of protein after 6 weeks in milk not containing chalk, and this appears to have been obtained at the expense, mainly, of the proteose-peptone fraction, which shows a corresponding decrease. Yeast extract apparently exerts no constant influence in this respect. Changes in the amino acid fraction are much smaller, sometimes showing a slight increase and sometimes a slight decrease. The two strains of *Str. cremoris* present a rather different picture. *Str. cremoris* I shows a slight increase in protein and a fairly large increase in amino acids, these increases being derived from the proteose-peptone fraction. *Str. cremoris* II shows a considerable decrease in the protein and proteose-peptone fractions, accompanied by a large increase in the amino acids. Possibly the difference in these two strains of the same species may be accounted for by the fact that strain II had been fairly recently isolated from starter, whereas strain I had been kept in artificial culture for a long time, and may have lost some of its proteolytic power in consequence. The mixed culture of streptococci shows a definite decrease in protein after 4 weeks, due apparently to its degradation to proteose-peptone or amino acids, both of which fractions show increases.

(5) Cultures containing chalk show a breakdown of protein in every instance. With both strains of *Str. lactis* and with *Str. cremoris* I it is considerable after 12 weeks. With *Str. paracitrovorus* it is only very slight after the same time, while with the mixture of streptococci it is considerable after 4 weeks. In each case loss of protein is accompanied by a corresponding increase in the proteose-peptone fraction, but the changes in the amino acid fraction are comparatively small and show either increase or decrease. There is a general tendency for protein breakdown to be greater in cultures containing yeast extract than in plain milk cultures and for amino acids to increase more in plain milk than in milk containing yeast extract. In fact, where decreases in amino acid content occur they are in cultures containing yeast extract, and it may be noted that in such cultures the original supply of amino acids is considerably greater than it is in plain milk. Such utilization of amino acids is, however, always small.

(6) The alcohol-formalin titrations show changes which are comparatively small and the frequent decreases in these values indicate the extent to which the amount of free amino nitrogen is being lessened by peptide linkage in the process of anabolism. The fact that increase or decrease in the proteose-peptone or in the amino acid fraction is often not accompanied by a corresponding increase or decrease in the alcohol-formalin titration of the whole culture or the alcohol-soluble portion respectively shows, too, that an increase in total amount of either of these fractions may be accompanied by a peptide linking between some of the constituents, thereby reducing the amount of free amino nitrogen.

(2) *Experiments with lactobacilli.*

The seven strains of lactobacilli used were *Lactobacillus plantarum* I (from Autumn grass silage), *L. plantarum* II (from acid molasses silage), *L. plantarum* III (from Spring grass silage), *L. casei* I (from Cheddar cheese), *L. casei* II (from molasses silage), *L. bulgaricus* (from Egyptian fermented milk) and *L. acidophilus* (stock culture). Their reactions in litmus milk media and in carbohydrate broths (determined when the cultures were freshly isolated) are shown in Table II. All cultures had been kept for some time in the laboratory before being used for these experiments. As with the streptococci data were obtained for individual strains and for a mixed culture containing *L. plantarum* I, *L. plantarum* II, *L. casei* I, *L. bulgaricus* and *L. acidophilus*. Typical figures for bacterial counts are shown in Tables VI and VII, figures for acid development in Table IX, alcohol-formalin titrations for two strains in Tables XI and XII, and a summary of the changes in nitrogen distribution

Table VI. *Lactobacillus plantarum* I

Bacterial counts per ml.

		Days								Weeks								
Age of culture		0	1	2	4	6	7	9	15	4	6	8	10	12	14	19	27	35
Without chalk	Y.E.	10^5	—	10^7	10^7	10^8	—	10^8	10^8	10^8	10^8	10^7	10^7	—	10^5	10^4	10^4	10^2
	No Y.E.	10^5	—	10^6	10^7	10^7	—	10^8	10^9	10^8	10^8	10^9	10^8	—	10^6	10^4	10^5	10^3
With chalk	Y.E.	10^5	10^7	10^8	10^{10}	—	10^{10}	—	10^{10}	10^9	—	10^8	—	10^7	—	—	—	—
	No Y.E.	10^5	10^6	10^7	10^{10}	—	10^{10}	—	10^9	10^9	—	10^9	—	10^8	—	—	—	—

Table VII. *Lactobacillus casei* I

Bacterial counts per ml.

		Days										Weeks					
Age of culture		0	1	2	3	4	6	7	9	12	14	4	8	12	17	27	36
Without chalk	Y.E.	10^7	10^7	—	10^8	—	10^8	—	10^9	10^9	10^{10}	10^9	10^8	—	10^6	10^4	10^3
	No Y.E.	10^7	10^8	—	10^9	—	10^8	—	10^8	10^9	10^8	10^9	10^8	—	10^7	10^5	10^4
With chalk	Y.E.	10^6	10^7	10^8	—	10^9	—	10^{10}	—	—	10^{10}	10^{10}	10^9	10^9	—	—	—
	No Y.E.	10^6	10^7	10^8	—	10^8	—	10^9	—	—	10^9	10^{10}	10^9	10^9	—	—	—

Table VIII. *Acid development by cultures of streptococci* (*ml.* N/10 *acid per* 100 *ml. culture*)

Strain	Medium	Age of culture (weeks) 0	2	4	6	10	12	14
Str. lactis I	Y.E.	0·0	—	—	92·0	—	103·8	—
	No Y.E.	0·0	—	—	87·2	—	95·0	—
Str. cremoris I	Y.E.	0·0	—	85·0	—	95·6	—	—
	No Y.E.	0·0	—	71·9	—	87·6	—	—
Str. cremoris II	Y.E.	0·0	69·0	—	87·0	93·4	—	—
	No Y.E.	0·0	63·7	—	82·8	78·6	—	—
Str. paracitrovorus	Y.E.	0·0	—	—	19·0	—	—	43·9
	No Y.E.	0·0	—	—	14·8	—	—	27·1
Leuconostoc mesenteroides	Y.E.	0·0	—	—	48·5	—	—	80·4
	No Y.E.	0·0	—	—	6·0	—	—	26·8
Mixed culture	Y.E.	0·0	86·0	91·5	—	—	—	—
	No Y.E.	0·0	75·0	75·5	—	—	—	—

Table IX. *Acid development by cultures of lactobacilli* (*ml.* N/10 *acid per* 100 *ml. culture*)

Strain	Medium	Age of culture (weeks) 0	2	4	8	18	19	20	25	27	34	36
L. plantarum I	Y.E.	0·0	58·7	91·1	114·7	—	129·9	—	—	135·3	—	—
	No Y.E.	0·0	27·6	47·9	68·2	—	97·9	—	—	107·3	—	—
L. plantarum II	Y.E.	0·0	64·6	65·4	97·9	—	—	115·5	—	134·8	—	141·3
	No Y.E.	0·0	27·1	42·5	58·9	—	—	85·6	—	104·4	—	117·4
L. plantarum III	Y.E.	0·0	6·5	9·5	—	21·6	—	—	45·8	—	60·8	—
	No Y.E.	0·0	6·4	8·1	—	15·0	—	—	19·2	—	50·9	—
L. casei I	Y.E.	0·0	79·6	97·3	137·7	—	—	142·9	—	150·5	—	152·8
	No Y.E.	0·0	36·6	49·1	63·4	—	—	86·0	—	105·2	—	127·6
L. casei II	Y.E.	0·0	62·6	58·3	87·8	—	—	110·2	—	130·7	—	139·1
	No Y.E.	0·0	21·0	30·4	46·1	—	—	61·2	—	88·6	—	100·6
L. bulgaricus	Y.E.	0·0	4·7	5·3	—	81·4	—	—	99·9	124·5	—	—
	No Y.E.	0·0	3·2	4·3	—	20·7	—	—	55·9	83·9	—	—
Mixed culture	Y.E.	0·0	105·7	170·9	—	—	—	—	—	—	—	—
	No Y.E.	0·0	75·6	153·6	—	—	—	—	—	—	—	—

Table X. *Streptococcus cremoris* I

Alcohol-formalin titrations (ml. *N*/10 per 100 ml.)

Age of culture (weeks)	Without chalk: Whole culture Y.E.	Without chalk: Whole culture No Y.E.	Without chalk: Alc. filtrate Y.E.	Without chalk: Alc. filtrate No Y.E.	With chalk: Whole culture Y.E.	With chalk: Whole culture No Y.E.	With chalk: Alc. filtrate Y.E.	With chalk: Alc. filtrate No Y.E.
0	31·5	27·9	13·2	8·4	33·2	25·3	11·6	7·0
2	—	—	—	—	15·8	12·0	10·6	7·9
4	34·5	23·4	13·5	5·3	12·0	7·9	14·8	8·1
8	—	—	—	—	18·6	14·0	14·1	9·4
10	35·7	27·0	15·9	11·0	—	—	—	—
12	—	—	—	—	23·5	17·1	15·2	10·4

is shown in Table XIII. Each figure is the average of duplicates. The following conclusions may be drawn:

(1) The maximum numbers attained are approximately the same in cultures with or without yeast extract, and the presence of chalk makes no difference in this respect.

(2) The viability of the lactobacilli is very much greater than that of the streptococci, so that even after 30–36 weeks in cultures without chalk there are still appreciable numbers present. Addition of yeast extract decreases the

Table XI. *Lactobacillus plantarum* I

Alcohol-formalin titrations (ml. *N*/10 per 100 ml.)

Age of culture (weeks)	Without chalk				With chalk			
	Whole culture		Alc. filtrate		Whole culture		Alc. filtrate	
	Y.E.	No Y.E.	Y.E.	No Y.E.	Y.E.	No Y.E.	Y.E.	No Y.E.
0	32·6	30·5	17·5	13·2	32·9	25·9	14·6	8·7
2	27·9	14·9	13·4	13·1	11·4	—	21·0	—
4	27·4	20·6	17·0	12·7	13·1	—	16·4	14·3
6	29·6	20·5	15·5	14·1	—	—	—	—
8	30·2	26·9	19·7	17·4	20·4	23·8	18·6	13·6
10	28·0	23·4	18·8	10·0	—	—	—	—
12	—	—	—	—	31·2	32·5	19·8	17·1
14	28·5	21·4	17·1	11·6	—	—	—	—
19	30·5	24·4	18·1	13·1	—	—	—	—
27	31·8	27·3	18·5	14·7	—	—	—	—
35	32·5	28·6	19·6	15·9	—	—	—	—

Table XII. *Lactobacillus casei* I

Alcohol-formalin titrations (ml. *N*/10 per 100 ml.)

Age of culture (weeks)	Without chalk				With chalk			
	Whole culture		Alc. filtrate		Whole culture		Alc. filtrate	
	Y.E.	No Y.E.	Y.E.	No Y.E.	Y.E.	No Y.E.	Y.E.	No Y.E.
0	33·8	29·4	15·6	12·9	31·6	25·3	12·3	9·1
2	30·4	15·5	18·0	15·0	10·5	11·1	19·9	6·3
4	27·8	16·9	15·4	13·3	15·5	23·1	15·0	12·6
6	24·0	20·0	12·8	9·3	—	—	—	—
8	33·8	25·0	14·5	9·6	20·5	28·9	14·1	15·4
12	—	—	—	—	29·2	34·4	14·0	17·8
20	25·9	25·3	12·4	7·5	—	—	—	—
27	32·5	26·6	16·4	13·3	—	—	—	—
36	35·4	28·9	18·2	15·3	—	—	—	—

viability, but only slightly. These observations are in accordance with the well-known acid-resisting character of the lactobacilli.

(3) Addition of yeast extract results in a very marked increase in the amount of lactic acid produced, and this applies whether the strain is one which forms a large or comparatively small amount of acid.

(4) In the cultures not containing chalk the nitrogen distribution figures for some of the lactobacilli show conditions which are similar to those found for the streptococci. That is to say, with *L. plantarum* I, *L. plantarum* II, and

L. casei II there is, after several weeks' incubation, an increase of protein, which has apparently been derived mainly from the proteose-peptone fraction, changes in the amino acid fraction being comparatively slight. Similar conditions obtain in the cultures of *L. casei* I and *L. bulgaricus* when yeast extract is present but the cultures in plain milk are different. *L. plantarum* III, on the other hand, exhibits definite proteolysis, accompanied by an increase in both the proteose-peptone and amino acid fractions. The mixed culture also shows proteolysis but here the proteose-peptone fraction is slightly decreased while the amino acids are considerably increased.

Table XIII. *Nitrogen distribution in cultures of lactic acid bacteria. Increase or decrease in each fraction at the end of the incubation period (mg. N per 100 ml. culture)*

Culture	Age (weeks)	Medium	Protein		Proteose-peptone		Amino acids, etc.	
			Y.E.	No Y.E.	Y.E.	No Y.E.	Y.E.	No Y.E.
Streptococci								
Str. lactis I	6	No chalk	+ 14·0	+23·6	− 9·8	−18·7	− 4·1	− 0·8
	12	Chalk	− 26·3	−21·2	+31·8	+20·5	− 5·5	+ 0·8
Str. cremoris I	6	No chalk	+ 2·0	+ 3·8	−12·3	−12·2	+ 10·3	+ 8·4
	12	Chalk	− 27·4	−18·3	+23·3	+ 9·0	+ 3·0	+ 9·3
Str. cremoris II	6	No chalk	− 24·2	−21·0	−20·9	− 5·8	+ 45·1	+26·8
Str. paracitrovorus	6	No chalk	+ 10·6	+10·8	− 8·2	−11·3	− 2·4	+ 0·5
	12	Chalk	− 1·1	− 1·7	+ 1·4	+ 2·1	− 0·4	− 0·4
Leuconostoc mesenteroides	6	No chalk	+ 18·6	+18·4	−17·8	−25·0	− 0·8	+ 6·6
Mixed culture	4	No chalk	− 8·0	− 6·2	+ 3·0	+ 3·8	+ 5·0	+ 2·4
	4	Chalk	− 32·1	−31·4	+34·9	+28·3	− 2·9	+ 3·1
Lactobacilli								
L. plantarum I	14	No chalk	+ 8·2	+ 5·2	− 9·9	− 8·2	+ 1·8	+ 3·0
	12	Chalk	− 37·0	−39·1	+19·3	+15·2	+ 17·6	+25·0
L. plantarum II	20	No chalk	+ 6·6	+ 6·6	− 8·4	− 9·0	+ 1·8	+ 2·5
	12	Chalk	− 42·9	−36·1	+19·5	+ 2·2	+ 13·4	+33·9
L. plantarum III	20	No chalk	− 26·4	−22·7	+13·3	+11·2	+ 13·1	+11·5
L. casei I	20	No chalk	+ 13·2	−22·8	−10·2	+ 6·9	− 3·0	+15·9
	12	Chalk	− 18·4	−54·8	+11·3	+15·4	− 2·9	+39·4
L. casei II	20	No chalk	+ 10·8	+ 4·4	−10·7	− 5·3	0·0	+ 0·9
L. bulgaricus	20	No chalk	+ 4·4	− 6·8	− 6·4	+ 1·8	+ 2·1	+ 5·1
L. acidophilus	4	Chalk	−112·7	−33·4	+ 9·5	+23·6	+102·1	+ 9·8
Mixed culture	4	No chalk	− 18·0	−10·6	− 4·9	− 4·5	+ 22·9	+15·1
	4	Chalk	−112·9	−72·8	+ 2·5	+12·7	+110·3	+60·1

In cultures containing chalk the four single species tested all show appreciable proteolysis, which in cultures of *L. acidophilus* is considerable after only 4 weeks' incubation. The nitrogen distribution in the mixed culture is comparable with that for *L. acidophilus*, except that when yeast extract is present proteolysis is greater in the mixed culture. In all cases there is an increase in proteose-peptone and often a very considerable increase in amino acids. In general the formation of amino acids in these neutralized cultures is much more characteristic of the lactobacilli than it is of the streptococci.

(5) The alcohol-formalin titrations show comparatively small changes for the cultures to which no chalk had been added and often there is a decrease in these figures. This corresponds with the anabolic process revealed by the nitrogen distribution figures. In cultures containing chalk, however, where there are large increases in the amino acid fractions in the nitrogen distribution figures, these are paralleled by very definite increases in the alcohol-formalin titrations of the alcohol-soluble portions of the cultures.

(6) With the exceptions of *L. acidophilus*, *L. plantarum* III and the mixed culture there is a tendency for the addition of yeast extract to result in the formation of smaller quantities of amino acids or in a negative value, compared with the same culture in plain milk. The three exceptions are all strongly proteolytic cultures.

Discussion of results

The picture suggested by all the figures obtained for the various cultures is that of concomitant synthesis and degradation of the proteins and their split products, corresponding to the dual role of the bacteria in (*a*) reducing the nitrogen sources provided to compounds which can be directly utilized, and (*b*) building up cell proteins. This is shown most obviously, perhaps, by *Str. cremoris* I, where increase of protein is accompanied by a fairly large increase in the amino acids, the proteose-peptone fraction having been used partly in synthesis and partly in degradation processes. Since in the majority of cultures, both of streptococci and lactobacilli, which are not neutralized by chalk, there is an increase of protein following prolonged incubation, while in the same cultures to which chalk has been added there is considerable breakdown of protein, it is evident that *p*H is one factor controlling the process, presumably through its effect on the proteolytic enzyme. Neutralization of the developed acid removes its inhibitory action on the latter with the result that protein breakdown exceeds protein synthesis.

On the other hand, it appears that other factors affect the process. Thus with two strains of lactobacilli (*L. casei* I and *L. bulgaricus*) the unneutralized cultures show increase of protein in presence of yeast extract and a decrease in its absence. Since with both of these strains addition of yeast extract has resulted in a very much greater development of acid, it may be that its influence on protein degradation is exerted indirectly through its effect on *p*H. It may be noted, in fact, that in several of the cultures neutralized with chalk protein breakdown is greater when yeast extract is present, though there is one noticeable exception to this—*L. casei* I. In most cases, too, yeast extract tends to reduce amino acid formation.

Apart from the influence of external factors cultures show an inherent individuality. Thus the marked protein degradation shown by *L. plantarum* III in milk not containing chalk is not, perhaps, surprising in view of the small amount of acid it forms, but this does not apply to *Str. cremoris* II, which produces considerable acid but is nevertheless markedly proteolytic.

The observation that, where increase of protein occurs, the proteose-peptone fraction has contributed mainly to it indicates that it is the higher products of protein decomposition, rather than the amino acids, which are utilized by the lactic acid bacteria in nutrition.

Davis & Mattick (18), working with broth cultures, found that acid production by streptococci was greatest with peptones or casein digests containing the higher fractions of protein decomposition, whereas with lactobacilli it was the lower fractions which most stimulated fermentation. The nitrogen distribution figures shown in this paper do not suggest any appreciable utilization of amino acids in milk by either streptococci or lactobacilli in the process of protein synthesis. It is possible, of course, that the picture may be different in earlier stages of growth than those investigated here, but if the amino acids were used extensively at first the process must have been followed in most cases by a compensating degradation of peptone while leaving the protein unchanged, an unlikely hypothesis which is not supported by the alcohol-formalin titrations after 2 weeks' growth.

Conclusions

1. The growth of streptococci or lactobacilli in milk results, with most species, in an increase of protein after several weeks' incubation. This is derived mainly from the proteose-peptone fraction, which shows a corresponding decrease. Changes in the amino acid fraction are usually very small.

2. Occasional species show an exception in this respect and result in protein degradation.

3. Mixed cultures of either streptococci or lactobacilli bring about marked proteolysis after 4 weeks.

4. Addition of chalk to the milk used for cultures results in considerable proteolysis. With the streptococci the protein is degraded mainly to proteoses and peptones, but with several species of lactobacilli appreciable quantities of amino acids are formed.

5. When yeast extract is added to the milk much more acid is developed by the cultures, though this does not appear to be accompanied by a marked increase in the maximum numbers of bacteria.

6. Yeast extract decreases the viability of cultures (particularly of streptococci) not containing chalk, presumably owing to the lethal effect of the extra acid produced. When chalk is present viability is not affected by yeast extract. The viability of lactobacilli is very much greater than that of streptococci.

7. Growth of these bacteria in milk results in concomitant synthesis and degradation of proteins and their split products, the latter process being inhibited by high acidity. Addition of chalk, by neutralizing the acid, removes this inhibitory effect and results in a preponderance of protein degradation.

8. Provision of an additional quantity of amino acids in the form of yeast extract usually results in a tendency to stimulate the anabolic process so that either smaller quantities of amino acids are produced or negative values

obtained after the growth of the cultures. The few exceptions to this are strongly proteolytic lactobacilli.

9. There is no evidence in these studies that proteolytic power of the lactic acid bacteria is linked with acid-producing power, though it is only fair to point out that there was not a large number of strains of any one species tested in this respect.

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REFERENCES

(1) v. Freudenreich, E. (1896–8). Quoted by Hucker, G. J. *Tech. Bull. N.Y. agric. Exp. Sta.* no. 89, 1922.
(2) Orla-Jensen, S. (1904). *Zbl. Bakt.* II Abt. **13**, 514.
(3) Barthel, C. (1915). *Zbl. Bakt.* II Abt. **44**, 76.
(4) Barthel, C. & Sandberg, E. (1919). *Zbl. Bakt.* II Abt. **49**, 392.
(5) Anderegg, L. T. & Hammer, B. W. (1929). *J. Dairy Sci.* **12**, 114.
(6) Barthel, C. & Sadler, S. (1928). *Trans. Roy. Soc. Can.* **22**, 233.
(7) Sherwood, I. R. & Whitehead, H. R. (1934). *J. Dairy Res.* **5**, 208.
(8) Kulp, W. L. & Rettger, L. F. (1924). *J. Bact.* **9**, 357.
(9) Sherwood, I. R. (1935). *J. Dairy Res.* **6**, 204 and 407.
(10) Orla-Jensen, S. (1919). *The Lactic Acid Bacteria.* Copenhagen.
(11) —— (1933). *J. Soc. chem. Ind., Lond.*, **52**, 374 T.
(12) Orla-Jensen, S. & Jacobsen, J. (1930). *Zbl. Bakt.* II Abt. **80**, 326.
(13) Allen, L. A. (1930). *J. Dairy Res.* **2**, 38.
(14) Rowland, S. J. (1938). *J. Dairy Res.* **9**, 30 and 42.
(15) —— (1937). *J. Dairy Res.* **8**, 1.
(16) Thomas, P. (1913). *C.R. Acad. Sci., Paris*, **156**, 2024.
(17) Allen, L. A. & Harrison, J. (1936). *Ann. appl. Biol.* **23**, 546.
(18) Davis, J. G. & Mattick, A. T. R. (1932). *J. Dairy Res.* **4**, 81.

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208. THE PART PLAYED BY BACTERIA IN THE REDUCTION OF METHYLENE BLUE IN MILK

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(With 4 Figures)

The methylene blue reduction test, either in its original or its modified form, is now being extensively used for the bacteriological grading of milk in many countries of Europe and in the United States of America. It is generally believed that the reduction time affords an index partly of the numbers of bacteria present in the milk and partly of their rate of metabolism. The exact mechanism of the reaction, however, is still in dispute. What part, for instance, is played by the natural reducing system of the milk, what part by the oxygen consumption of the organisms, what part by the production of reducing systems of their own by the bacteria, and what part by other factors, remains uncertain. It was the purpose of the present investigation to inquire a little more closely into the mechanism of the test, paying particular attention to the rôle of bacterial growth and metabolism in the reduction of methylene blue.

Observations on the reducing activity of different bacteria in milk

Technique of the methylene blue reduction test

The modified method of performing the reduction test described by Wilson *et al.* (1) was used. Briefly, this technique was as follows: Each sample of milk, after thorough mixing, was poured into a calibrated test-tube with a 10 ml. graduation mark. The methylene blue solution was added in 1 ml. quantities by a special delivery pipette. The tubes were closed with sterile rubber bungs, inverted, and placed in a covered water-bath at 37–38° C. They were inverted every half-hour until the commencement of decolorization. The end-point was considered to have been reached when the methylene blue was completely decolorized, or was completely decolorized except for a zone of 5 mm. at the top or bottom of the milk column. Two control tubes were put up with heated milk, one containing methylene blue, the other saline, in order to assist in judging the commencement and the completion of decolorization. The time of reduction was taken as the interval between the moment of incubation of the tubes in the water-bath and the attainment of the end-point. With one exception the detailed technique employed was essentially that which is laid down for the official grading of raw milk in this country (2). The exception concerned the brand of methylene blue tablets used. During the early part of

the work the standard English tablets were not available, and the original Danish tablets, supplied by Messrs Blauenfeldt and Tvede of Copenhagen, were therefore used throughout.

Attention may here be drawn to an error that occurred in the report by Wilson *et al.* (1). It is stated there that the final concentration of methylene blue was 1/300,000. This figure was supplied by the manufacturers of the Danish tablets themselves. A subsequent examination, however, of the tablets undertaken by the British Dye Corporation of Imperial Chemical Industries, Ltd., revealed the fact that by the titanous chloride method of titration each tablet contained approximately 16 mg. of pure methylene blue. The final concentration, therefore, of the dye in the milk tubes was about 1/550,000. This result was confirmed by a series of observations made in this laboratory in which a biological method of standardization was used, preparations of specially purified methylene blue supplied by Imperial Chemical Industries, Ltd., being taken as standard.

Reduction tests with raw milks

Preliminary experiments were carried out with many different samples of raw milk to determine the types of flora present at reduction. The milks were obtained from various outlying farms through the courtesy of United Dairies, Ltd., and were allowed to stand at room temperature for some hours before being tested. After reduction of the dye had taken place, a loopful of each milk was plated on to MacConkey agar and milk agar plates. Single colonies of the predominating organisms were isolated and identified as far as possible. Examples of the type of result obtained are given in Table I. The predominating

Table I. *Predominant organisms at time of reduction in various samples of raw milk incubated at* 37–38° C.

Sample no.	Reduction time hr. and min.	Predominant organisms
1	11·00	*Staph. aureus*, coliform, and *Achromobacterium*
2	9·45	*Staph. aureus* and *Achromobacterium*
3	9·45	*Staph. aureus*
4	9·28	*Staph. aureus*
5	9·00	*Staph. aureus*, micrococci and streptococci
6	9·00	*Staph. aureus* and *Achromobacterium*
7	7·00	Coliform
8	7·00	Coliform and micrococci
9	7·00	Coliform
10	6·45	Coliform
11	5·15	Coliform
12	5·00	Coliform and staphylococci
13	5·00	Coliform and *Staph. aureus*
14	5·00	Coliform and *Staph. aureus*
15	4·30	*Staph. aureus* and streptococci
16	3·40	Coliform
17	2·35	*Ps. pyocyanea* and *Achromobacterium*
18	2·30	Micrococci and streptococci
19	2·30	Coliform and streptococci
20	1·35	Very mixed culture
21	1·30	Very mixed culture
22	1·13	Very mixed culture
23	0·35	Very mixed culture
24	0·17	Very mixed culture

organism or organisms are recorded in the last column; other bacteria were subsidiary. The conclusions drawn from these results were as follows:

(1) At 37° C. the same few species of organism assumed predominance for every milk having a reduction time greater than 2–3 hr.

(2) Those milks in which *Staphylococcus aureus* prevailed reduced slowly.

(3) Milks reducing in a shorter time usually contained a predominating flora of coliform organisms.

(4) A *Streptococcus* (not identified) quickened the reaction when present together with organisms which normally reduced slowly (see milks 15 and 18 in the table).

(5) Those milks which reduced in less than 2 hr. contained a flora so mixed that it was impossible to single out any one predominating organism.

Reduction tests with pure cultures of bacteria

The organisms isolated in the way described in the previous section, together with some pathogenic members of the *Salmonella* group, were examined for their ability to reduce methylene blue in raw and pasteurized milk.

For this purpose "sterile" milk was used. This consisted of milk withdrawn aseptically by means of a catheter from one quarter of the healthy udder of a cow. Its average plate count at 37° C. on milk agar was 280 per ml. At the time of inoculation the milk was usually not more than 2 hr. old. Pasteurized "sterile" milk was prepared by exposing the raw milk to a temperature of 63° C. for 30 min. in a water-bath, followed by immediate cooling.

The strains to be tested were prepared as follows: The organisms were grown for 18–20 hr. at 37° C. on a convenient medium such as heart agar, or milk agar, or Hartley's agar. The growth was then washed off with sterile quarter-strength Ringer's solution made up with glass-distilled water, and the suspension was standardized by opacity to a strength equivalent to 1000 million *Bact. coli* per ml. In some instances, when the organism differed considerably in size from that of *Bact. coli*, its dimensions were measured by a calibrated eyepiece micrometer, its volume calculated, and an adjustment made so as to ensure that the total volume of bacterial protoplasm in the suspension was approximately equal to that in the *coli* standard. The suspension was diluted to the required degree, and added to the milk by means of a sterile dropping pipette.

Two sets of observations were made. In the first set small inocula were used, giving a final concentration in the milk of about 10,000 organisms per ml. In the second set the inocula were much larger, giving a final concentration of about 10,000,000 organisms per ml. Representative results are recorded in Table II. Several points of interest emerge from this table. It will be noticed that there was a considerable variation between different organisms in the rate at which they reduced methylene blue. The coliform and *Salmonella* group of organisms, with the exception of *Bact. typhosum*,

Table II. *Observations on reduction time of individual strains in raw and pasteurized "sterile" milk inoculated with* 10,000 *and* 10,000,000 *total organisms per ml.*

	Reduction time in hr. and min.			
	10,000 org. per ml.		10,000,000 org. per ml.	
Organism	Raw	Past.	Raw	Past.
Bact. coli I*	6·25	7·20	2·40	2·40
Bact. coli II	17·15	14·00	3·20	3·20
Intermediate I	16·00	13·03	2·30	2·30
Intermediate II	14·00	11·40	2·45	2·45
Bact. aerogenes I	—	—	2·40	2·40
Bact. aerogenes II	7·40	7·40	—	—
Staph. aureus	10·05	10·05	3·15	3·20
Staph. albus	—	—	4·35	4·35
Str. lactis†	—	—	2·20	2·04
Str. faecalis, type 2	—	—	8·35	9·35
Micrococcus	>20·00	>20·00	7·40	5·15
Micrococcus	17·40	14·10	6·50	4·55
Chromobacterium	15·50	13·00	—	—
Achromobacterium	17·25	>20·00	—	—
Pseudomonas pyocyanea	>20·00	>20·00	—	—
Bact. typhosum (BS)	—	—	4·35	4·10
Bact. typhosum (Watson)	—	—	4·25	3·30
Bact. typhosum (Giglioli)	—	—	4·55	3·30
Bact. paratyphosum A	—	—	2·15	3·30
Bact. paratyphosum B	—	—	3·15	2·45
Bact. paratyphosum C	—	—	2·15	3·30
Bact. typhi-murium	—	—	2·40	2·40
Bact. enteritidis	—	—	2·40	2·40

* The coliform organisms are classified in accordance with the scheme suggested by Wilson *et al.*(1).

† For classification of lactic acid and faecal streptococci, see p. 42.

reduced most rapidly; staphylococci and *Bact. typhosum* occupied an intermediate position; while *Ps. pyocyanea* and organisms of the *Micrococcus*, *Chromobacterium* and *Achromobacterium* groups, reduced very slowly or not at all. The relative inactivity of this third group of organisms was probably attributable to the fact that their optimum temperature is below 37° C. In tests carried out at room temperature it can be shown that these organisms are quite active reducers. With regard to the streptococci, organisms of the *lactis* type reduced rapidly, while organisms of the *faecalis* type (see p. 42) were much less active.

It will be further noticed that the results obtained depended to a considerable extent on the size of the inoculum. With small inocula there was a striking difference between the reduction times of *Bact. coli* I on the one hand and of *Bact. coli* II and the intermediate types on the other, while with large inocula the difference was very much less. It is difficult to explain this except on the supposition that small numbers of *Bact. coli* I are able to grow more rapidly in milk than certain other members of the coliform group.

On the whole the reduction time tended to be much the same in pasteurized as in raw milk, though a few irregularities occurred.

One further point may be noticed, which is not evident from the table. Observations on the length of time elapsing between the commencement of decolorization and complete reduction showed that this varied with different organisms. With the coliform group the interval was short and decolorization occurred simultaneously in all parts of the tube. With slower reducing organisms, on the other hand, such as some of the staphylococci and micrococci, decolorization often occupied an hour or more and tended to occur irregularly throughout the tube.

The rate of oxygen uptake by pure cultures of bacteria in milk in relation to their reduction time

To determine how far it is possible to explain the differences in reduction time between different groups of organisms by the rate at which they take up oxygen from the medium and so allow the natural reducing system of the milk to come into effective operation, observations were made with a Barcroft differential manometer on the oxygen-absorbing power of various organisms growing in milk under aerobic conditions.

The apparatus was calibrated in accordance with the formula given by Dixon (3). "Sterile" milk, either raw or pasteurized, was added in 3 ml. quantities to each flask. The left-hand flask served as a control; the right-hand flask was inoculated with suitable numbers of the organism under investigation. No buffer solution was added, since the milk itself appeared to have a sufficient buffering power. The central compartment of each flask contained 0·3 ml. of a 10% solution of caustic potash, together with a small roll of filter paper, 4 × 1·5 cm., to afford a large surface for the absorption of any CO_2 evolved.

Preliminary observations

Before making observations on oxygen absorption, it was clearly necessary to exclude the possibility that the results might be vitiated by the evolution of hydrogen from the cultures, particularly from cultures of coliform organisms. For this reason a series of preliminary experiments was undertaken. Briefly, these consisted in growing strains of *Bact. coli* and of *Bact. aerogenes* in autoclaved milk contained in a round-bottomed flask closed with a rubber bung through which passed an exit tube fitted with a stopcock. The flasks were incubated in a water-bath at 37–38° C. and shaken continuously. At intervals the gaseous contents were withdrawn and analysed in a Haldane apparatus modified for the detection of combustible gases (Haldane (4)). With an inoculum of 100,000 per ml. no measurable amount of hydrogen was found to be produced by either of these organisms until the culture had been incubated for over 6 hr. and until the total number of organisms present exceeded 600 million per ml.

Since our observations on oxygen absorption were to be made with "sterile" milk, in which growth is considerably slower than in autoclaved

milk, and were to be limited to periods of up to 6 hr., it was concluded that no appreciable error was likely to be expected from this source.

Starting with coliform organisms, experiments were then made with pure cultures in "sterile" raw milk, using an inoculum of about 50,000 per ml. in order to simulate conditions existing in milk of fairly high quality. To our surprise very little growth occurred for some hours and practically no oxygen was taken up. With larger inocula, however, of approximately 2,000,000 per ml., rapid growth occurred and considerable quantities of oxygen were absorbed. Inquiry into various possible reasons that might be responsible for the failure of small inocula to grow in the Barcroft flasks resulted in our tracing the main cause to the presence of the caustic potash in the central chamber. When this was replaced by Ringer's solution normal growth occurred. From the work of Gladstone *et al.* (5) it seemed clear that growth would not occur unless more than a minimal amount of CO_2 was present. With small inocula the rate of absorption of CO_2 by the caustic potash in the shaken flasks was so rapid that growth was almost completely inhibited. Against our will, therefore, we were compelled to use relatively large inocula.

Final technique adopted

After various other points had been investigated, the final technique adopted was as follows: "Sterile" milk was added in 3 ml. quantities to Barcroft flasks. The bacterial suspensions were prepared in the way described on p. 37 and standardized by opacity. The counts were frequently checked by means of a Helber slide adapted to dark-ground work (Wilson (6, 7)). To the right-hand flask of each Barcroft manometer was added 0·05 ml. of the suspension by means of a calibrated dropping pipette so as to give a final concentration of about 15 million total organisms per ml. The flasks were incubated in a mechanically stirred water-bath at 37–38° C. and shaken continuously for 6 hr. Manometric readings were taken every hour. Tubes containing 10 ml. of milk and 1 ml. of methylene blue solution were inoculated simultaneously with the same number of organisms, and the reduction time observed in the usual way. Breed smears were made on the milk in the Barcroft flasks at the end of 6 hr. Instead of making replicate observations on the same organism on one day, single observations were made on six different organisms on the same day, in order to avoid as far as possible the disturbance caused by variations in the bacteriostatic power of the milk from day to day. Each set of organisms was studied on three different days, and the mean value for the triplicate observations on each organism was calculated.

Rate of oxygen absorption in raw and pasteurized milk

Observations on a number of common bacteria found in milk are recorded in Table III. Taking the last four columns first, it will be noticed that there was a great difference in the amount of oxygen taken up by different organisms. The coliform bacteria took up a far larger amount than any of the others. On

Table III. *Relationship between reduction time and oxygen uptake of individual strains in raw and pasteurized "sterile" milk at 37–38° C. (Inoculum about 15 million total organisms per ml.)*

Organism	Reduction time hr. and min.		O_2 uptake in c.mm. at time of reduction		O_2 uptake in c.mm. after 6 hr.	
	Raw	Past.	Raw	Past.	Raw	Past.
Bact. coli I	2·15	2·57	21	55	434	512
Bact. coli II	2·42	3·17	25	50	272	322
Intermediate I	1·57	2·20	20	25	436	874
Intermediate II	2·15	2·28	21	39	837	997
Bact. aerogenes I	2·02	2·07	37	44	618	926
Staph. aureus	4·38	5·03	17	28	49	41
Staph. albus	4·13	4·30	39	37	109	110
Staph. citreus	5·18	6·10	40	62	61	60
Ps. pyocyanea	4·38	>6·00	51	>126	133	126
Str. lactis	3·23	3·35	18	13	40	37
Str. faecalis (H 91)	3·27	2·52	31	12	49	38
Str. faecalis (S iv)	4·12	>6·00	12	0	24	0
Str. faecalis (H12)	>6·00	>6·00	>15	>16	15	16
Str. faecalis F 22	>6·00	>6·00	—	—	0	0
Str. pyogenes, group A	>6·00	>6·00	—	—	0	0
Str. agalactiae, group B	>6·00	>6·00	>40	>28	40	28
Haemolytic streptococcus, group C	5·45	>6·00	—	—	0	0
Micrococcus	>6·00	>6·00	>87	>105	87	105
Micrococcus	>6·00	>6·00	—	—	0	0

—=Not observed. 0=No O_2 absorbed.

the whole the staphylococci and one group of micrococci took up more than the streptococci. The haemolytic streptococci—groups A and C—and some of the faecal streptococci took up least of all.

No close relationship appeared to exist between the rate of reduction of methylene blue and the amount of oxygen taken up in 6 hr. It is true that the rapidly reducing coliform organisms took up the largest quantity of oxygen, but careful study of the table reveals a number of discrepancies with the other organisms. *Staph. aureus*, for example, which absorbed a relatively small amount of oxygen, reduced methylene blue in $4\frac{1}{2}$–5 hr., while one strain of *Micrococcus* and *Ps. pyocyanea*, which took up two to three times the amount of oxygen, failed to reduce even in 6 hr. Another example is afforded by *Str. lactis* and one of the faecal streptococci, which in both raw and pasteurized milk took up a relatively small amount of oxygen and reduced in 3–$3\frac{1}{2}$ hr., while *Str. agalactiae*, which absorbed much the same amount of oxygen, completely failed to reduce methylene blue in the 6 hr. period of observation. It will be further noticed that with the coliform organisms the majority of the oxygen was taken up after the time at which methylene blue was decolorized in the reduction tubes.

At the time of decolorization of the methylene blue the amount of oxygen that had been absorbed varied considerably with different organisms. While *Bact. coli* I, for example, in raw milk had taken up only 21 c.mm., one strain of *Micrococcus* had taken up four times this amount and had still not caused

reduction. It must, of course, be realized that the highly aerobic conditions in the shaken Barcroft flasks were very different from the relatively anaerobic conditions in the motionless corked reduction tubes. There is no reason to suppose, however, that the oxygen avidity of the organisms differed significantly under these two sets of conditions. The real difference lay in the amount of oxygen available to satisfy their requirements. We regard it therefore as justifiable to assume that the rate of oxygen absorption in the Barcroft flasks afforded an index of the rate of oxygen absorption in the reduction tubes.

Breed smears showed that with nearly all organisms clumps were present in both raw and pasteurized milk after 6 hr. It was originally intended to make viable counts at intervals on the cultures in the Barcroft flasks and calculate the amount of oxygen taken up by a single cell during the lag and logarithmic phases of multiplication. The amount of clumping, however, that occurred in milk soon rendered such an attempt fruitless, since it was clear that the plate count was affording both a gross and an irregular underestimate of the real number of viable organisms present. Often the clumps were so dense that even a count of the individual organisms in a Breed smear was quite impossible.

Incidentally, observations were made on the oxygen-absorbing power of two strains of *Bact. coli*, one of which formed clumps during growth in milk, the other of which did not. With an inoculum of 1,500,000 organisms per ml. the non-clumping strain took up 27 c.mm. of oxygen in 6 hr. and 415 c.mm. in 9 hr. The corresponding figures for the clumping strain were 36 and 355 c.mm. Allowing for the variations that occur between different strains, there is little in these figures to suggest that the amount of oxygen absorbed is greatly affected by the degree of aggregation of the organisms (see also p. 48).

A further set of observations was made with raw "sterile" milk, using a number of strains of each organism. The results are summarized in Table IV and represented graphically in Fig. 1. On the whole, the figures confirm those of Table III. It will be noted, however, that *Staph. aureus* was more active in reducing methylene blue and in taking up oxygen than appeared previously.

With regard to the non-haemolytic streptococci we endeavoured to differentiate between the *lactis* and *faecalis* types on the basis of the criteria suggested by Sherman & Stark (8). The attempt was not altogether successful, since one strain of presumably *lactis* type used for a cheese starter, though thermolabile and not growing in skimmed milk at 45° C., nevertheless grew well on lactose agar of *p*H 9·6 and on lactose agar containing 6·5 % sodium chloride. Again, a strain of presumably *faecalis* type, isolated from adult human faeces, showed no growth in skimmed milk at 45° C., even after 48 hr. The four *lactis* strains and four of the *faecalis* strains, which we have labelled for convenience type 1, reduced methylene blue fairly rapidly, while six *faecalis* strains, which we have designated type 2, failed to reduce within the period of observation.

Table IV. *Relationship between average reduction time and average oxygen uptake of pure cultures of bacteria in raw "sterile" milk at 37–38° C. (Inoculum about 15 million total organisms per ml.)*

Organism	No. of strains examined	Average reduction time in hr. and min.	Average O_2 uptake in c.mm. at time of reduction	Average O_2 uptake in c.mm. after 6 hr.
Bact. coli I	10	2·34	37	490
Bact. coli II	1	2·42	25	272
Intermediate I	10	2·49	43	583
Intermediate II	1	2·42	32	527
Bact. aerogenes I	2	2·14	45	742
Bact. cloacae	2	2·27	38	501
Staph. aureus	7	3·46	58	186
Staph. albus	3	4·44	40	63
Staph. citreus	6	5·03	42	61
Str. lactis	4	3·27	8	25
Str. faecalis, type 1	4	3·03	19	67
Str. faecalis, type 2	6	>6·00	>10	10
Str. pyogenes, group A	3	>6·00	>7	7
Str. agalactiae, group B, type 1	7	5·20	17	23
Str. agalactiae, group B, type 2	3	>6·00	>14	14
Haemolytic streptococci, group C	4	5·25	10	14
Micrococcus, type 1	2	4·43	23	45
Micrococcus, type 2	4	>6·00	>55	55
Aerobic spore-bearers	6	6·25	475	391

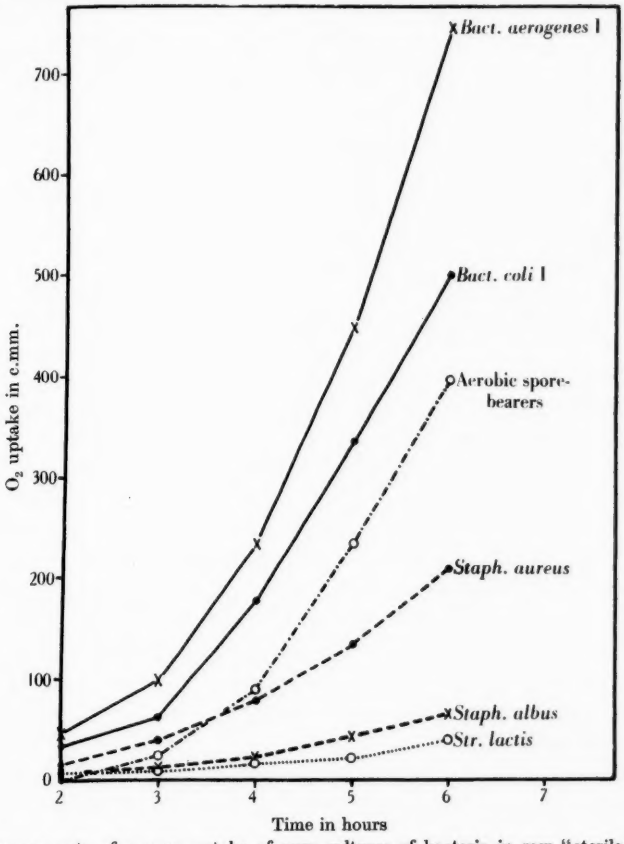

Fig. 1. Average rate of oxygen uptake of pure cultures of bacteria in raw "sterile" milk at 37–38° C. (Inoculum about 15 million total organisms per ml.)

Of the haemolytic streptococci group A was almost devoid of activity, while group C had an average reduction time of under 6 hr. and took up a small amount of oxygen. Special interest attaches to group B—the so-called *Str. agalactiae*—which is responsible for bovine mastitis. Certain workers (Malcolm & Leitch (9)) have regarded this organism as practically inactive towards methylene blue. Examination of Table IV shows, however, that seven of the ten strains, which we have designated type 1, had an average reduction time of under $5\frac{1}{2}$ hr.

Attention may be drawn to the behaviour of the aerobic spore-bearers. These organisms took up a large amount of oxygen, yet had a long reduction time. In this respect they seemed to resemble the behaviour of *Micrococcus lysodeikticus* described by Hewitt (10).

Oxidation-reduction potentials of pure cultures of bacteria in milk

It was clearly desirable to learn something of the oxidation-reduction potentials established in milk cultures, and to bring these into relation with the reducing and oxygen-absorbing capacities of the same organisms. This was rendered possible by the co-operation of Mr Twigg, of the Department of Biochemistry, who kindly consented to make observations on potential-time curves of the main types of organisms that we were studying.

The apparatus and technique used were the same as those already described in the report by Wilson *et al.* (1). Briefly, electrode vessels containing 50 ml. of raw "sterile" milk were inoculated with a bacterial suspension in a quantity sufficient to give a final concentration of 15 million total organisms per ml. The vessels were incubated in an oil bath at 37° C., and measurements of the oxidation-reduction potential of the cultures were made at intervals by means of a platinum electrode. A vessel containing uninoculated milk, through which oxygen-free nitrogen was bubbled, served as a control. All vessels contained methylene blue in the same concentration as in the ordinary reduction test. In each experiment simultaneous observations were made on the oxygen-absorbing capacity of the organisms by the Barcroft differential manometer in the way already described (p. 40).

The organisms tested comprised *Bact. coli* I, *Bact. aerogenes* I, *Str. lactis*, and six members of the aerobic spore-bearing group. Several attempts made with staphylococci proved unsatisfactory owing to the poor growth of these organisms. Typical examples of the results obtained are illustrated in Figs. 2 and 3. In the control tube through which nitrogen was bubbled continuously complete reduction of the methylene blue occurred in $1\frac{1}{2}$–2 hr. at an E_h value of about +35 mV. (see Fig. 3). The limiting potential reached at a *p*H of 6·4–6·6 was usually −180 to −200 mV.

The cultures of *Bact. coli* and *Bact. aerogenes* (Fig. 2) were characterized by a precipitous fall of potential, which appeared to correspond with the beginning

of the oxygen uptake. The limiting potential E_h −350 mV. attained by these organisms was very much lower than that in the control milk, corresponding in fact to the hydrogen over-voltage potential. Presumably this was due to the fact that hydrogen was being produced from the lactose in the milk. Boyd & Reed (11), it may be remembered, actually observed the liberation of molecular hydrogen in glucose broth cultures of *Bact. coli* when the potential

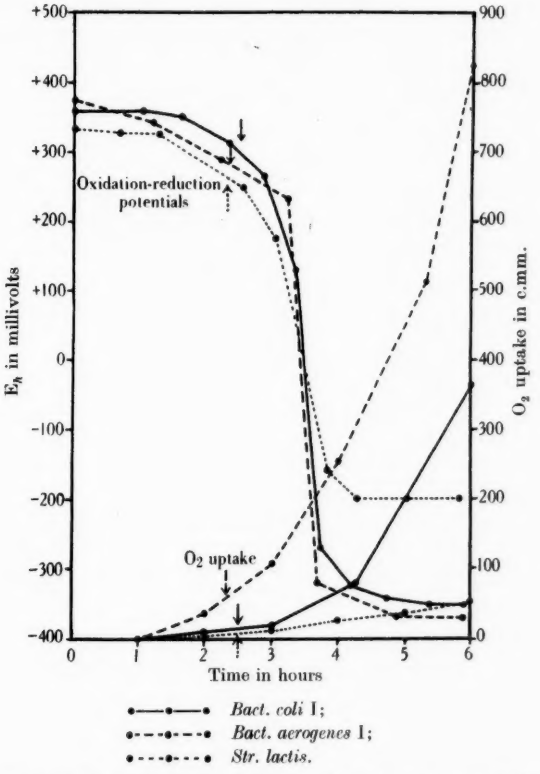

Fig. 2. Oxidation-reduction potentials and oxygen uptake of pure cultures of bacteria in raw "sterile" milk incubated aerobically at 37–38° C. The arrows on the oxidation-reduction potential curves refer to the time of reduction of methylene blue in the milk. (Inoculum about 15 million total organisms per ml.)

had reached its lowest point. We are not concerned here with the exact mechanism by which members of the *coli-aerogenes* group produce their low potentials, but it seems not unlikely that, as Twigg (12) suggests, "activated" hydrogen may be formed under enzyme action and completely reduce the lactoflavine in the milk. In any case large quantities of molecular hydrogen are liberated, which can combine with oxygen dissolved in the milk, thus accounting for the high oxygen absorption of these organisms.

In cultures of *Str. lactis* (Fig. 2) the limiting potential reached was never below that of the "sterile" control milk. This organism does not appear to possess the enzymes necessary to produce molecular hydrogen from any substrate in milk, and consequently it is unable to lower the final potential to a value more negative than that of the milk itself, i.e. −180 to −200 mV. Oxygen absorption is very small, and what oxygen is taken up must presumably be due to the oxidation of substances other than hydrogen.

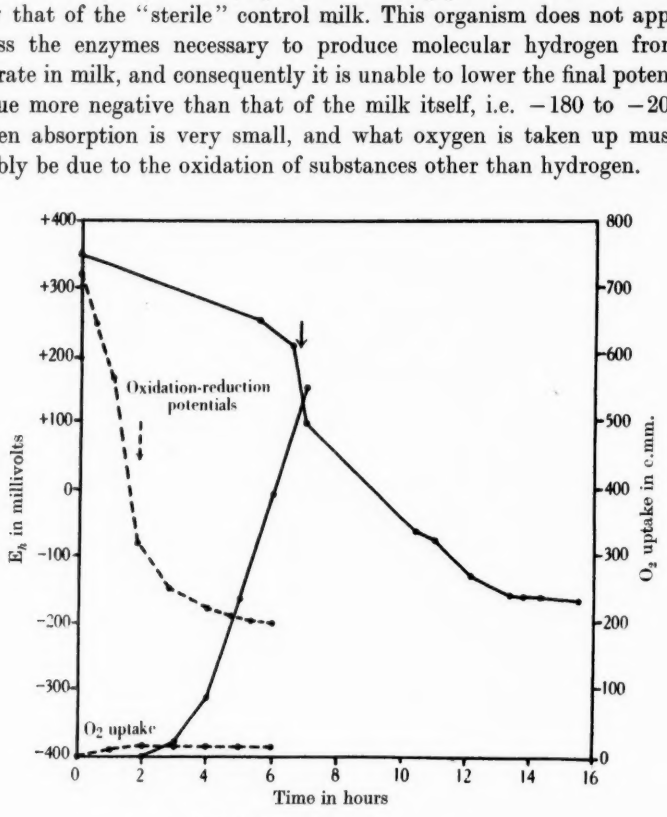

Fig. 3. Average oxidation-reduction potentials and average oxygen uptake of pure cultures of aerobic spore-bearing organisms in raw "sterile" milk incubated aerobically, and of uninoculated raw "sterile" milk incubated in nitrogen at 37-38° C. The arrows on the oxidation-reduction potential curves refer to the time of reduction of methylene blue in the milk. (Inoculum about 15 million total organisms per ml.)

Six strains of aerobic spore-bearers (Fig. 3) were studied, five of which had been recently isolated from milk. Compared with the coliform and *Str. lactis* strains, the oxidation-reduction potential fell very slowly and did not reach its limiting value of −170 mV. for about 15 hr. Most striking was the relatively high oxygen uptake before reduction of the methylene blue occurred. For instance, by the time the dye was decolorized and the oxidation-reduction potential had fallen to between +250 and +280 mV., *Str. lactis* had taken up about 10 c.mm., *Bact. coli* I about 25 c.mm., *Bact. aerogenes* I about 60 c.mm., and the aerobic spore-bearers about 450 c.mm. of oxygen.

It will be observed that, though methylene blue was not completely reduced in the control milk until the E_h value had fallen to about +35 mV., in the culture tubes it was reduced at a very much higher level—in the neighbourhood of +300 mV. The explanation of this is not altogether clear. Perhaps the E_h at the surface of the bacteria, where the dye was being reduced, was considerably lower than that recorded by the electrodes in the milk. Whatever the explanation, there seems no doubt that the rapid fall in potential, with the consequent reduction of methylene blue, bore very little relation to the amount of oxygen that had been absorbed.

OBSERVATIONS ON FACTORS CAUSING AGGLUTINATION AND INHIBITING THE MULTIPLICATION OF BACTERIA IN MILK

Whatever the respective parts played by the absorption of oxygen and the production of a reducing system by organisms in milk, it is evident that the time taken by any one type of organism to reduce methylene blue must depend on its rate of growth. It was considered desirable, therefore, to make a few observations on the growth-inhibiting factors that are known to be present in fresh raw milk.

Since 1894, when Hesse (13) drew attention to the inhibitory effect of milk on the growth of *Bact. typhosum* and *V. cholerae*, numerous observations have been made on this property by different workers. After inoculation of the milk, plate counts of certain organisms often show a decline for some hours before multiplication occurs. Rosenau & McCoy (14) suggested that this might be due to the presence of agglutinins rather than of actual bactericidal substances in the milk. By the plate count technique it is not easy to differentiate between the effect of these two substances. Chambers (15) was of the opinion that both agglutinins and growth-inhibitory substances existed in milk, and that they had no relation to each other, except that they were both destroyed by heating to 80–90° C. for 2 min. *Bact. lactis acidi*, for example, was clumped but not inhibited by milk, whereas with *Bact. coli* the reverse occurred.

The most extensive observations were made by Jones (16) and Jones & Simms (17, 18). Working with mastitis and with pyogenic streptococci, these workers obtained evidence of the existence of a non-specific growth-inhibitory factor in raw milk and whey. It was destroyed by heating to 80° C. for 20 min., and could be filtered readily through a Berkefeld V but not through a Berkefeld W candle. It appeared to be of protein nature, and it could be extracted from whey in a relatively pure form—"lactenin". This substance had 200–500 times the activity of dried skim milk.

Observations on the agglutinating factors in milk

Our own observations can be briefly recounted.

The *agglutinating effect of milk* was determined in two ways. (*a*) Milk was inoculated with about 10 million organisms per ml., and incubated in a water-bath at 37–38° C. Breed smears were made at half-hourly intervals, and the

number and size of the bacterial clumps recorded. (*b*) Milk whey was prepared according to the method of Hall & Learmonth (19), involving the use of carbon tetrachloride and rennet. The clear whey was serially diluted with saline, mixed with an equal volume of bacterial suspension standardized to 1000 million per ml., and incubated in a water-bath at 55° C. overnight. The tubes were examined for macroscopic agglutination the following morning. In the preparation of whey from autoclaved milk calcium was added to assist in the formation of the clot.

The following results were obtained:

(1) Six out of fourteen strains of *Bact. coli* I, and all strains of intermediate I, intermediate II, and *Bact. aerogenes* I (two strains of each type tested) formed clumps in "sterile" raw and pasteurized, but not in autoclaved milk. Clumping was evident within 2 or 3 hr. of inoculation. *Staph. aureus* and *Str. faecalis* behaved similarly.

(2) The addition of whole raw milk, or of cream or whey prepared from "sterile" raw milk, to cultures in autoclaved milk restored the clumping property of the milk. The size of the clumps depended on the amount of the material added. The addition of autoclaved whey to autoclaved whole milk did not have this effect.

(3) With *Bact. coli* strains, but not with other members of the coliform group, clumping was closely associated with motility.

(4) There appeared to be no relation between clumping and the mucoid or non-mucoid character of the colonies formed by strains of *Bact. coli* I on milk agar.

(5) Eight non-clumping strains of *Bact. coli* I had an average reduction time in "sterile" raw milk of 2 hr. 20 min., and in "sterile" pasteurized milk of 2 hr. 11 min. The corresponding reduction times of six clumping strains were 2 hr. 35 min. and 2 hr. 19 min. respectively. These differences are certainly not significant, and it would appear that with coliform bacilli the degree of aggregation has but little effect on the rate at which they are able to reduce methylene blue.

(6) Removal of the cream from "sterile" raw and pasteurized milk was without action on the clumping effect, showing that the butter fat is not responsible for the aggregation of organisms that occurs in milk cultures.

(7) By the tube technique agglutinins in low titre (1/8–1/16) were found in raw milk whey for many strains of *Bact. coli* I, for strains of intermediate and *aerogenes* type, and for such members of the *Salmonella* group as *Bact. typhosum*, *Bact. typhi-murium*, and *Bact. enteritidis*.

(8) With all the organisms that were tested the two methods used yielded similar results, indicating that the substance in milk whey which causes agglutination is probably the same as that which is responsible for the formation of clumps in whole milk.

(9) The clumping or agglutinating factor was destroyed by exposure to a temperature of 70–75° C. for 1 hr. This suggests that it is of protein nature.

Observations on the growth-inhibitory factor in milk

The rate of multiplication of various milk bacteria was estimated in the following way. Tubes containing 10 ml. of the medium under investigation—usually "sterile" raw milk, "sterile" boiled or autoclaved milk, or whey prepared from these milks by rennet without the use of carbon tetrachloride—were inoculated with between 500 and 2000 viable organisms per ml. The cultures were incubated in a water-bath at 37° C., and viable counts were

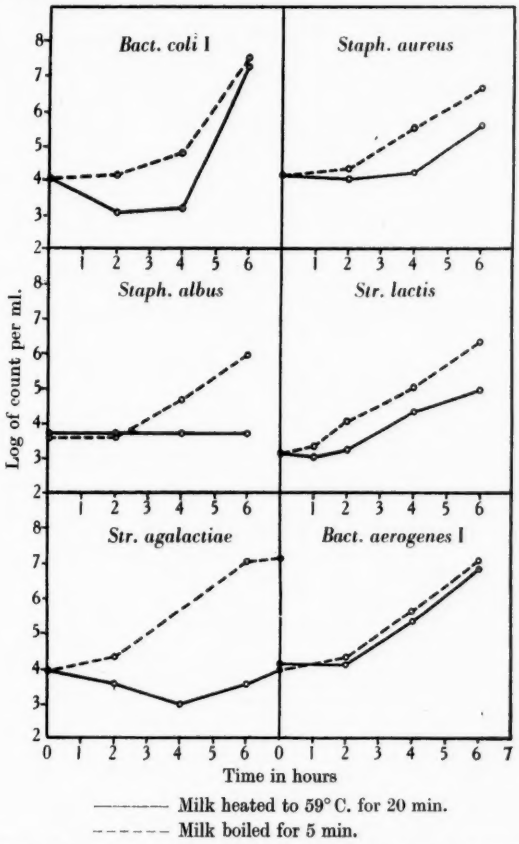

Fig. 4. Rate of growth of pure cultures of different organisms in "sterile" milk heated to 59 and to 100° C.

made after 0, 1, 2, 4, 6, and sometimes 8 hr. The results may be summarized as follows:

(1) With several organisms, such as *Bact. coli* I, *Staph. aureus*, *Staph. albus*, *Staph. citreus*, *Str. lactis* and *Str. agalactiae*, and sometimes, though not always, with *Bact. aerogenes*, the onset of multiplication was delayed for 2–6 hr. or more in raw milk, but not in raw whey, or in whole milk or whey that had been boiled for 5 min. or autoclaved at 110° C. for 20 min. (Fig. 4).

(2) The possibility that this delay in multiplication was apparent rather than real, being due to agglutination rather than to true inhibition of growth, could not be excluded, but two facts seemed to argue against it: (*a*) the delay in multiplication occurred equally with non-clumping and with clumping strains of *Bact. coli* I; (*b*) the number of organisms inoculated was so small that the chances of their meeting each other and forming clumps was probably not very high—particularly with the non-motile species.

(3) The growth-inhibitory factor was destroyed by exposure to a temperature of 70–75° C. for 1 hr.

From these observations it would appear that there is a clumping or agglutinating factor and a growth-inhibitory or bacteriostatic factor present in "sterile" raw milk. From the variety of different organisms on which these factors act, it is probable that they are non-specific. They resemble each other in being destroyed by heat at 70–75° C. for 1 hr., and are presumably both of protein nature. Whether they are identical it is impossible to say, but the probability is that they are not. In the first place the clumping factor is present in both raw whole milk and whey, whereas the bacteriostatic factor is present in raw whole milk but not in whey. Secondly, some strains of *Bact. coli* I are affected by the bacteriostatic but not by the clumping factor.

It must be pointed out that the use of the term growth-inhibitory factor is perhaps not strictly justified by our evidence. The method we were using, namely, the plate count, measures only multiplication. It does not afford any index of the growth in size that is known to occur before division sets in. To estimate growth as a whole as distinct from multiplication, either a microscopical method must be used, or else some technique such as the methylene blue reduction test, which affords a measure of the metabolic activity of the organisms (Topley & Wilson (20)).

Wilson *et al.* (1), who compared the plate count and the reduction time of milks at regular intervals after inoculation, found that, though the plate count often remained stationary or actually decreased for 2–3 hr. or more, the reduction time fell from the moment of incubation. We repeated these experiments with coliform bacteria and with *Staph. aureus*, using "sterile" raw milk inoculated with 50,000 to 200,000 living organisms per ml., and obtained similar results. Occasionally, however, a strain of *Bact. coli* or of *Bact. aerogenes* showed no increase in plate count for 6 hr., and no fall in reduction time for about 3 hr., suggesting that a real growth-inhibitory factor was present in the milk.

It may be concluded that there is present in fresh clean raw milk some factor which is capable of preventing bacterial multiplication for a variable length of time. How far there is a real growth-inhibitory factor is still uncertain. Not until further observations have been made on the plate count and reduction time of "sterile" milk inoculated with different numbers of organisms will it be possible to speak definitely on this point. There is a certain amount of evidence, however, to suggest that a real growth-inhibitory factor

does exist, but that it is capable of dealing only with small numbers of organisms and is more effective with some species and strains than with others.

In this connexion it may be mentioned that the clumping factor, which is present in raw milk and whey, and which acts on certain organisms but not on all, may be responsible for a decrease in the apparent rate of bacterial multiplication as estimated by the plate count, but does not appear to have any significant effect on the reduction time.

Discussion

The primary object of the work described in this paper was to get a closer insight into the mechanism by which methylene blue is reduced in the presence of growing cultures of different bacteria in milk. Several investigators have studied this problem. Since a fairly complete review of their work has recently been compiled by Wilson *et al.* (1), there is no need here to do more than refer to papers that have appeared within the last two or three years.

Matuszewski (21), and Matuszewski *et al.* (22, 23, 24), paid particular attention to the relation between the rate of bacterial growth and the rate of reduction of methylene blue in sterilized milk cultures of *Str. lactis*. They found a positive correlation between the rate of reproduction and the intensity of hydrogen activation. They obtained some evidence that the decolorization of methylene blue followed the exhaustion of the dissolved oxygen in the milk, and they expressed the opinion that after all the oxygen had been used up by the bacteria methylene blue acted as a hydrogen acceptor. Like Zelikow (25) before them, they found that the rate of reduction of methylene blue could be used to measure approximately the numbers of growing bacterial cells in the medium.

Jackson (26) found that cows' milk withdrawn aseptically and maintained under anaerobic conditions reduced methylene blue in 1–30 min. He suggested that lactoflavin might be responsible for the natural reducing activity of the milk. How far this surmise is true does not concern us here; but we are concerned with his further opinion that bacterial action has no necessary relation to the reduction of methylene blue. To quote his exact words; "Bacteria may play but an insignificant part in the reduction of methylene blue in milk, though their deoxygenating effect may be of influence in the commercial application of the test." This expression of opinion is surprising in view of the fact that Jackson apparently made no observations whatever on the reducing systems present in milk containing actively growing bacteria.

If it could be shown (*a*) that the natural reducing system in milk varied enormously from cow to cow or from time to time in the milk of the same cow, and (*b*) that the natural reducing system was sufficiently active to bring about reduction of methylene blue under aerobic conditions, there might be some foundation for Jackson's opinion. Experience, however, in this laboratory during the past six years has failed to show any considerable variation in the activity of the natural reducing system present in milk. At no time has

"sterile" raw milk been found capable of reducing methylene blue under aerobic conditions. Moreover, as Twigg(27) points out, the total reducing capacity of "sterile" milk towards methylene blue is very small, whereas the total reducing capacity of milk in which active bacterial multiplication is occurring is often very great. For example, in a series of four experiments made at different times "sterile" milk through which oxygen-free nitrogen was bubbled continuously reduced methylene blue in a final concentration of 1/550,000 in about 1 hr. Increasing the concentration by 50% to 1/370,000 lengthened the reduction time to about 1 hr. 50 min. When the concentration was doubled to 1/225,000 complete reduction failed to occur in 4 hr. On the other hand, milk containing large numbers of bacteria proved very much more active. Thus in one experiment 21 hr. agar slope cultures were washed off with $N/4$ Ringer solution, standardized by opacity, and added to "sterile" raw milk in quantities sufficient to give a final count of 10,000 million organisms per ml. Methylene blue was then added in varying concentrations, and the tubes were incubated at 37–38° C. The normal strength (1/550,000) of methylene blue was reduced almost immediately. Sixty-four times the normal strength (1/86,000) was reduced in the following times: *Staph. aureus* 20 min.; *Bact. coli* I, intermediate I, and *Bact. aerogenes* I 45–90 min.; aerobic spore-bearer 75 min. Higher concentrations of dye were not tried, but Thornton & Hastings(28) state that reduction of methylene blue in milk by bacteria has been reported in concentrations as high as 1/3000. It seems clear, therefore, that the total reducing capacity of bacterial suspensions is very much greater than that of the natural reducing system of milk.

Jensen(29) found that *Bact. coli*, lactic acid streptococci, micrococci, and aerobic spore-bearers were among the most rapidly reducing organisms in milk, while those organisms that failed to ferment lactose reduced more slowly. Streptococci were observed to reduce methylene blue more rapidly in pasteurized or sterilized milk than in raw milk, whereas with *Bact. coli* the reverse occurred. Jensen concluded that the rate of reduction was related to the metabolic activities of the organisms in the milk. Discussing the mechanism of the reaction, he suggests that methylene blue serves as a catalyst to facilitate the transfer of dissolved oxygen to the growing bacteria. He supposes, in fact, that the methylene blue, acting as a hydrogen acceptor, is reduced by bacterial metabolic products to the leuco compound, and that this is immediately converted back to the coloured form by the dissolved oxygen in the medium. This alternate reduction and oxidation continues until most of the oxygen molecules are exhausted and the methylene blue remains decolorized.

If Jensen's hypothesis were correct, it should be possible to demonstrate that the rate of oxygen uptake by organisms growing in milk is hastened by the addition of methylene blue. We carried out a number of experiments to test this point. Using the Barcroft differential manometer, we measured the rate of respiration of various organisms growing in pure culture in "sterile" raw milk, and in milk to which methylene blue had been added in the same

concentration as that used in the reduction test. Neither with actively growing nor with "resting" organisms did the presence of methylene blue make any observable difference to the rate of oxygen uptake. It would obviously be unjustifiable on the basis of these experiments to deny the catalytic action of methylene blue, but it is equally obvious that they afford it no support. That methylene blue acts as a hydrogen acceptor is perfectly clear; whether it has any action beyond this must remain doubtful for the present.

Without question the most widely held theory of methylene blue reduction is that advanced by Barthel(30) in 1917. Barthel maintains that the decolorization of the dye occurs in two stages; first, the removal of the dissolved oxygen by the growing bacteria, and secondly, the reduction of the dye by the natural reducing system present in the milk. Wilson *et al.*(1), who made a very thorough study of the methylene blue reduction test, agree to some extent with Barthel's theory, but they enlarge on the second phase of the reaction. They believe that the production of the negative potential necessary to bring about the decolorization of methylene blue is attributable not only to the natural reducing system of the milk, but to reducing substances formed by the organisms themselves. Indeed they bring a considerable amount of evidence to suggest that, at any rate with some organisms, the reducing system formed by the bacteria is of far greater importance than the natural reducing system of the milk.

Our own observations were designed to test both the first and the second part of Barthel's theory. The results we obtained lead us to make the following relevant remarks:

(1) The amount of oxygen taken up by different organisms before methylene blue is reduced varies considerably. In Table III (p. 41), for example, *Staph. aureus* growing in raw milk had taken up 17 c.mm. of oxygen at the time of reduction, while a *Micrococcus* that had taken up over five times this amount had still not reduced methylene blue. Or again, in Table IV (p. 43) the group C haemolytic streptococci had taken up only 10 c.mm. of oxygen before they reduced methylene blue in 5½ hr., while the aerobic spore-bearers, which took up nearly fifty times this amount, did not reduce for 6½ hr. That small differences should occur in the absolute amount of oxygen absorbed would not be surprising, since the rate of absorption, with consequent differences in the time available for further solution of oxygen in the milk, varies from organism to organism; but that differences should occur of the magnitude actually observed seems unlikely if the preliminary absorption of oxygen represented the sole part played by bacteria in the reduction of the dye.

(2) In some experiments with certain organisms, when small inocula were used, both the amount of oxygen taken up and the time of reduction of methylene blue were greater in pasteurized than in raw milk, whereas with other organisms the reverse occurred. For example, reference to Table III (p. 41) shows that a strain of *Bact. coli* I absorbed nearly three times the amount of oxygen in pasteurized as in raw milk yet took half an hour longer

to reduce, while a strain of *Str. faecalis* absorbed only about a third of the amount of oxygen in pasteurized as in raw milk yet took half an hour less to reduce.

(3) There appears to be no constant relationship between the amount of oxygen absorbed by a given type of organism and the rate at which reduction occurs. Taking the total oxygen uptake in 6 hr., the reactions of the organisms we studied can be indicated roughly in the following way (Table V).

Table V. *Relationship between amount of oxygen consumed in* 6 *hr. and rate of reduction of methylene blue in raw "sterile" milk*

Type of organism	O_2 uptake	Rate of reduction
Coliform	+ + + +	+ + + +
Str. lactis *Str. faecalis*, type 1	+	+ + +
Staph. aureus	+ +	+ + +
Staph. albus *Staph. citreus* *Micrococcus*, type 1	+	+ +
Str. agalactiae, type 1 Haemolytic streptococci, group C	±	+
Aerobic spore-bearers	+ + +	±
Micrococcus, type 2	+	±
Str. pyogenes *Str. agalactiae*, type 2 *Str. faecalis*, type 2	±	±

It is true that members of the coliform group, which take up the largest amount of oxygen, are also the most rapid reducers, but *Str. lactis* and some faecal streptococci, which take up only about a tenth of the amount of oxygen (see Table IV), reduce nearly as rapidly. Again, the aerobic spore-bearers, which take up a relatively large amount of oxygen, reduce more slowly than many strains of haemolytic streptococci which take up only a fraction of this amount.

(4) On the other hand, so far as our limited observations go, there does seem to be a fairly high correlation between the rate of the fall in the oxidation-reduction potential of the culture and the rapidity with which methylene blue is reduced. Fig. 2 and Table IV show that this holds true for organisms of the coliform group and for *Str. lactis*. As regards staphylococci and micrococci, Table IV, taken in conjunction with the time-potential curves recorded by Wilson *et al.* (1), shows that these organisms form no exception to the rule. Further confirmation is afforded by the behaviour of the aerobic spore-bearers depicted in Fig. 3. In spite of their relatively large oxygen uptake, these organisms bring about only a very slow fall in the oxidation-reduction potential.

(5) Though, as pointed out in (2) above, the reduction time under certain conditions may be longer in pasteurized than in raw milk, examination of Table II (p. 38) shows that the reduction time with most organisms is much the same in pasteurized as in raw milk. Since the natural reducing system present

in raw milk is destroyed by pasteurization, it seems clear that reduction occurring in pasteurized milk cultures must be due to reducing substances formed by the growing organisms. If the natural reducing system played any considerable part in the reduction of methylene blue in cultures growing in raw milk, we should expect either that the dye would not be reduced at all in pasteurized milk cultures or not for very much longer than in raw milk cultures. The fact that the reduction time is usually the same in pasteurized as in raw milk cultures indicates that the reduction is caused almost entirely by the organisms themselves.

(6) That the natural reducing system present in milk is not necessary for the reduction of methylene blue is further evident from the fact that thick suspensions of some bacteria will reduce methylene blue as rapidly in Ringer solution as in milk, or even more rapidly. Thus in one experiment agar slope cultures of various organisms were washed off with $N/4$ Ringer solution, standardized by opacity, and diluted with $N/4$ Ringer solution to contain about 5000 million organisms per ml. A duplicate set of cultures was washed off with "sterile" raw milk, and the resulting suspensions were diluted with "sterile" raw milk to the same extent. Methylene blue was added to each of the suspensions, which were then incubated at 37–38° C. in a water-bath. With strains of *Bact. coli* I and II, intermediate I and II, and *Bact. aerogenes* I and II reduction occurred almost at once in the "Ringer" and the "milk" tubes. In another experiment reduction occurred in 15–17 min. in the "Ringer" and in 19–34 min. in the "milk" tubes. With a strain of an aerobic spore-bearing organism the corresponding times were 15 and 29 min.

Moreover, the practically instantaneous reduction of methylene blue that sometimes occurs when the dye is added to a milk approaching the souring point suggests that a far more active reducing system must be operative than that occurring naturally in the milk.

(7) As already recorded on p. 52, suspensions of various types of organisms were found capable, when added to "sterile" raw milk in numbers approximating to 10,000 million per ml. and incubated aerobically, to reduce 64 times the normal concentration of methylene blue in 20–90 min.; while "sterile" raw milk by itself was incapable even under anaerobic conditions of reducing twice the normal concentration of dye in 4 hr. It is true that the number of organisms far exceeded that normally present in milk at the time of reduction, but it must be remembered that they were more or less in a "resting" phase, and not actively growing as they are under the ordinary conditions of the reduction test.

For the reasons we have just set out it seems to us very doubtful whether Barthel's theory can be accepted any longer—at least in its entirety. While not denying that preliminary absorption of oxygen will favour the establishment of a negative oxidation-reduction potential, or that the natural reducing system of the milk may play some part in milk cultures of organisms whose

reducing activities are extremely low, it seems to us that far and away the most important factor in the reduction of methylene blue in milk cultures of actively growing bacteria is the formation of reducing substances by the organisms themselves. In our view the oxygen absorption is incidental rather than essential. As rapidly as reducing substances are formed hydrogen is donated, either directly as in the coliform group, or indirectly through a dehydrogenase system, and taken up probably simultaneously, though at different rates, by the methylene blue and by the dissolved oxygen. Since (*a*) the nature of the reducing substances, and the rate at which they are formed, almost certainly vary with different types of organisms; (*b*) the conditions required for the absorption of oxygen and those required for the reduction of methylene blue are probably quite different, as manifested for example by organisms containing cytochrome; (*c*) the amount of oxygen diffusing into the medium will depend partly on the length of the reduction time; and (*d*) the differential acceptance of hydrogen by oxygen and methylene blue will be governed, at least to some extent, by the concentrations of these substances in the milk—it is not difficult to understand why the amount of oxygen absorbed before reduction should vary so greatly with different organisms.

The reduction of methylene blue appears to be determined not, as Barthel supposes, by the natural reducing system of the milk, which comes into operation after the dissolved oxygen has been absorbed by the bacteria, but essentially as the result of the sum total of metabolic reactions proceeding at the cell surface of bacteria growing in the milk. This conclusion seems to us to be in consonance with that reached by Hewitt (10) in his discussion on the mechanism of bacterial reductions.

Summary and conclusions

1. In good quality raw milk reducing methylene blue at 37–38° C. members of the coliform group and *Staph. aureus* were among the commonest organisms present at the time of reduction. Other types of staphylococci, micrococci, and certain streptococci were not infrequently present in smaller numbers. In any given milk one type of organism tended to assume predominance.

2. Using the modified test described by Wilson *et al.* (1), various organisms isolated from milk at the time of reduction were examined for their rate of decolorization of methylene blue when growing in pure culture in raw "sterile" milk (milk freshly drawn from the healthy udder under aseptic conditions and having a plate count on milk agar at 37° C. of 200–300 per ml.). The most rapidly reducing organisms belonged to the coliform group. Following them, in decreasing order, came *Str. lactis* and some of the faecal streptococci, *Staph. aureus*, *Staph. albus*, *Staph. citreus*, some micrococci, group C haemolytic streptococci and some strains of *Str. agalactiae*, and aerobic spore-bearers.

3. Using the Barcroft differential manometer, the same organisms were tested for their power of absorbing oxygen when grown under highly aerobic

conditions in raw "sterile" milk at 37–38° C. With any given organism there appeared to be very little relationship between the rate of oxygen absorption in Barcroft flasks and the rate of reduction of methylene blue in corked tubes. Taking the amount of oxygen absorbed at the time of reduction, it was found, for instance, that the relatively slow reducing group C haemolytic streptococci took up 10 c.mm., the rapidly reducing *Str. lactis* 8 c.mm., the very rapidly reducing coliform organisms about 40 c.mm., and the slow reducing aerobic spore-bearers 400–500 c.mm. With the coliform group the greatest amount of oxygen in the Barcroft flasks was absorbed after reduction had occurred in the corked tubes. This was presumably due to the evolution of nascent hydrogen, which combined immediately with the available oxygen.

4. Electrode time-potential drifts were measured for coliform organisms, *Str. lactis*, and six members of the aerobic spore-bearing group growing under aerobic conditions in raw "sterile" milk at 37° C. The limiting potential (E_h −350 mV.) attained by members of the coliform group was considerably below that reached by uninoculated raw "sterile" milk incubated anaerobically (E_h −180 to −200 mV.), while that reached by *Str. lactis* and the aerobic spore-bearers was approximately equal to that of the uninoculated milk, i.e. E_h −180 to −200 mV. With the coliform organisms and *Str. lactis* the oxidation-reduction potential fell rapidly and reduction of methylene blue occurred at a time when only a very small amount of oxygen had been absorbed, while with the aerobic spore-bearers the oxidation-reduction potential fell slowly and reduction did not occur till a large amount of oxygen had been absorbed. Taking these potential measurements in conjunction with those of other workers, it seems probable that there is a fairly high correlation between the rate of the fall in the oxidation-reduction potential of the culture and the rapidity with which methylene blue is reduced.

5. Examination of raw "sterile" milk showed that it contained two factors, one of which was responsible for the clumping or agglutination of growing organisms, the other of which had a bacteriostatic effect inhibiting bacterial multiplication for a variable length of time. The first factor was present in whole milk and whey, the second only in whole milk. Both factors were destroyed by exposure to a temperature of 70–75° C. for 1 hr. The clumping factor appeared to have very little effect on the rate of oxygen absorption or of methylene blue reduction, but greatly diminished the plate count. The bacteriostatic factor inhibited bacterial multiplication as measured by the plate count. Whether it had a true growth-inhibitory effect as measured by the reduction test, i.e. whether it delayed the growth in size and active metabolism that precede cell division, was not definitely ascertained, but some evidence was obtained to suggest that this effect might be operative under certain conditions.

6. The mechanism by which methylene blue is reduced in milk containing actively growing bacteria is discussed in some detail. Reduction appears to be determined not, as Barthel supposes, by the natural reducing system of the

milk coming into operation, after the dissolved oxygen has been absorbed by the bacteria, but almost entirely as a result of the metabolic reactions proceeding at the cell surface of the bacteria themselves. Preliminary oxygen absorption is believed to be incidental rather than essential, while the part played by the natural reducing system of the milk is regarded as almost negligible except in milk containing organisms whose reducing powers are very low.

7. It is concluded, therefore, that the methylene blue reduction test, as carried out in practice, affords a good index of the extent of bacterial metabolism in the milk.

We are indebted to the Milk Marketing Board for a grant in aid of this work; to United Dairies, Ltd., for a supply of raw milk that was needed for certain purposes; to Dr F. C. Minett for arranging for a constant supply of "sterile" raw milk throughout the inquiry; and to Mr R. S. Twigg for his helpful co-operation in making a number of oxidation-reduction potential measurements on pure cultures of bacteria.

REFERENCES

(1) Wilson, G. S., Twigg, R. S., Wright, R. C., Hendry, C. B., Cowell, M. P. & Maier, I. (1935). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 206.
(2) (1937). *Memo.* 139/*Foods. Min. Hlth, Lond.*
(3) Dixon, M. (1934). *Manometric Methods.* Camb. Univ. Press.
(4) Haldane, J. S. (1935). *Methods of Air Analysis*, 4th ed. London: Griffin and Co., Ltd.
(5) Gladstone, G. P., Fildes, P. & Richardson, G. M. (1935). *Brit. J. exp. Path.* **16**, 335.
(6) Wilson, G. S. (1922). *J. Bact.* **7**, 405.
(7) —— (1926). *J. Hyg., Camb.*, **26**, 150.
(8) Sherman, J. M. & Stark, P. (1934). *J. Dairy Sci.* **17**, 525.
(9) Malcolm, J. F. & Leitch, R. H. (1936). *Scot. J. Agric.* **19**, 321.
(10) Hewitt, L. F. (1931). *Biochem. J.* **25**, 1452.
(11) Boyd, E. M. & Reed, G. B. (1931). *Canad. J. Res.* **4**, 605.
(12) Twigg, R. S. (1938). Personal communication.
(13) Hesse, W. (1894). *Z. Hyg. InfektKr.* **17**, 238.
(14) Rosenau, M. J. & McCoy, G. W. (1908). *Bull. U.S. hyg. Lab.* no. 41, 449.
(15) Chambers, W. H. (1920). *J. Bact.* **5**, 527.
(16) Jones, F. S. (1926). *J. exp. Med.* **43**, 451.
(17) Jones, F. S. & Simms, H. S. (1929). *J. exp. Med.* **50**, 279.
(18) —— —— (1930). *J. exp. Med.* **51**, 327.
(19) Hall, I. C. & Learmonth, R. (1933). *J. infect. Dis.* **52**, 27.
(20) Topley, W. W. C. & Wilson, G. S. (1936). *The Principles of Bacteriology and Immunity*, 2nd ed. London: Ed. Arnold and Co.
(21) Matuszewski, T. (1935). *Roczn. Nauk. rol.* **34**, 403.
(22) Matuszewski, T., Neyman, J., Pijanowski, E. & Supinska, J. (1935). *Lait*, **15**, 1057.
(23) Matuszewski, T. & Pijanowski, E. (1935). *Roczn. Nauk. rol.* **35**, 1.
(24) Matuszewski, T. & Supinska, J. (1935). *Roczn. Nauk. rol.* **35**, 249.
(25) Zelikow, J. (1906). *Zbl. Bakt.* **42**, 476, 570, 669.
(26) Jackson, C. J. (1936). *J. Dairy Res.* **7**, 31.
(27) Twigg, R. S. (1937). *J. Soc. chem. Ind., Lond.*, **56**, 129.
(28) Thornton, H. R. & Hastings, E. G. (1929). *J. Bact.* **18**, 293.
(29) Jensen, H-O. (1937). *Zbl. Bakt.* II Abt. **96**, 110.
(30) Barthel, C. (1917). *Z. Untersuch. Nahr.- u. Genussm.* **34**, 137.

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STUDIES IN MASTITIS

By J. G. DAVIS, J. McCLEMONT AND H. J. ROGERS

SYNOPSIS

Introduction—scope of investigation.

I. *The routine diagnosis of mastitis.*

Sampling—enrichment experiments—treatment of samples—technique of plating—examination of plates—choice of media—advantages and disadvantages of blood media—cultural confirmation of suspected colonies—number of tests required—cost of examination.

II. *A comparison of methods for the detection of subclinical mastitis caused by* Str. agalactiae.

(1) Plating of centrifuged deposits—(2) crystal violet aesculin agar (i.e. Edwards's medium without blood)—(3) plain blood agar—(4) milk agar—(5) the methylene blue reduction test—(6) the catalase test—(7) the rennet test—(8) the "growth of starter" test—(9) the brom-cresol-purple test—(10) the incubation test—(11) the titratable acidity test—(12) clinical examination.

III. *Mastitis in relation to udder counts.*

Low counts obtained with mastitis-free cows—high counts with some infected cows—value of milk agar in control work.

IV. *Mastitis in relation to the methylene blue reduction test.*

Importance as the methylene blue test now official—the rôle of mastitis streptococci, "secondary invaders" and cells in the milk—value in diagnosis—effect of storage of samples.

V. *Mastitis in relation to cheese-making.*

Aspects of mastitis most important to cheese-makers, viz. (1) growth of starter, (2) clotting with rennet, (3) bacterial off-taints, (4) changes in enzymes of milk, (5) changes in vitamin and growth-factor content—mastitis and the rennet test—total count and the rennet test—description of amplified rennet test—interpretation—chemical composition of mastitis milk—changes in enzyme content—faults: (1) effect on cheese body—(2) significance in "red spot"—(3) mastitis and slow starter—the rennet test and the starter test—mastitis and vitamin content—the starter test and the methylene blue test—total count and the starter test.

VI. *General observations, summary and conclusions.*

Relation of incidence of mastitis with age of the cow—effect of age on "udder count"—correlation of stage of lactation with mastitis and "udder count". Summary to all papers. Only two types of method—very simple tests on farm and bacteriological plating—laboratory indirect methods not worth doing except for special purposes.

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209. STUDIES IN MASTITIS

I. THE ROUTINE DIAGNOSIS OF MASTITIS

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INTRODUCTION

IN recent years the attention of those interested in all aspects of the dairy industry has been focused upon the subject of mastitis, particularly the chronic streptococcal type. On account of the wide distribution of this disease and its adverse effect upon milk and milk products, it has become urgently necessary for the producer, distributor and manufacturer to be able to recognize mastitis in the cow and its effect upon the milk. It is, therefore, desirable that all those interested in dairying should be as fully informed upon the subject as possible. Many simple tests for the detection of mastitis have been described and are in common use, although so many papers have been published showing their limitations that it is surprising that workers should continue to use them (1, 2, 3, 9). Opportunity has been afforded to the writers to make an extensive examination and comparison of such tests, with the main object of finding out if any simple test, or combination of simple tests, is really satisfactory for the diagnosis of chronic streptococcal mastitis. The enormous literature upon mastitis (much of it uncritical and, therefore, of little use) makes an adequate summary impossible, even if it were desirable in a paper of this nature, but reference may be made to a useful summary by Munch-Petersen (1).

It will be sufficient to say that the work of Minett *et al.* (2–11) has been taken as a basis for the present investigation, which was primarily concerned with methods of detection and the economic consequences of mastitis.

Over 6000 tests have been made upon about 300 cows in milk from six herds in three counties. These tests have yielded over 12,000 observations, so that the results are sufficiently numerous and random to give statistically significant correlations and conclusions.

CRITERION OF MASTITIS

It is well known that the udder of a cow, or the milk drawn from it, may show abnormalities without yielding appreciable numbers of the causative organism, and vice versa. Further, even where an udder is infected, repeated tests may be necessary before the organism is detected. Since the chronic

type of mastitis caused by *Str. agalactiae* (Minett's group I) is responsible for 90–99% of cases, is the only known contagious form of the disease and frequently cannot be detected by non-bacteriological methods, the interest of the producer who is concerned with eradication naturally centres on this type. Other forms are usually clinical and so less insidious. The presence of *Str. agalactiae* in the milk has, therefore, been taken as the criterion of this common form of mastitis. Group II (Minett) streptococci(11) were encountered on only a few occasions.

TECHNIQUE

Sampling

After some preliminary experiments, including tests on samples sent by post, the following method was adopted and found to be very satisfactory. Ordinary boiling tubes (large test-tubes) comfortably holding about 50 ml. were plugged, sterilized and labelled, and taken in a light three-ply frame to the farm by car about an hour before afternoon milking. Brom-cresol-purple paper tests were carried out immediately on the fore-milk. During milking early mid-milk samples were taken by the milker into the tubes, i.e. about half a pint was withdrawn from each quarter before taking the sample. The milker's hands were washed with soap and water before milking each cow. Usually a mixed sample from all four quarters was taken, but on some occasions single quarter samples were taken. If any quarter was described as "bad", it was always sampled separately. Afternoon samples were found to give lower counts and much cleaner plates generally. Rejection of the fore-milk gives a better sample of the general milk content of the udder, since the number of contaminants in the fore-milk is higher than that in the mid-milk (cf. Table I). It would seem, however, that this is not a very important point, as the earliest milk may contain both a higher number of mastitis streptococci and a correspondingly larger number of the miscellaneous flora. This is illustrated in Table I.

ENRICHMENT ATTEMPTS

The possible enrichment of mastitis streptococci in the samples by incubation with blood, serum, autolysed yeast, sucrose (which is strongly fermented by *Str. agalactiae*) and with crystal violet was investigated. After addition of various amounts of these substances the samples were incubated for 24 hr. at 37° C. and then plated in blood agar and in Edwards's medium. The results are summarized in Table II.

It will be seen that the best results were obtained with 20% blood and 20% serum. The difficulty of obtaining blood may, however, militate against the use of serum and blood for enrichment purposes, although if microscopic examination is used for diagnosis the method is valuable.

Table I. *Numerical gradient of flora in the udders of infected cows. Count per ml. of milk*

		Blood agar				Crystal violet aesculin blood agar				Milk agar	
		Milk		Deposit		Milk		Deposit		Milk	
Cow	Sample	Total count	"Strepto-cocci"	Total count	"Strepto-cocci"	Total count	"Strepto-cocci"	Total count	"Strepto-cocci"	Total count	"Strepto-cocci"
1	A	500	500	330	330	400	400	300	300	800	700
	B	300	300	300	300	500	500	300	300	700	600
	C	700	700	30	30	600	600	30	30	800	800
	D	300	300	20	20	500	500	20	20	700	700
2	A	>1000	>1000	300	300	300	100	300	300	⩾1000	⩾1000
	B	>1000	>1000	270	100	500	500	—	—	>1000	>1000
	C	500	500	100	100	2	0	10	0	400	200
	D	>1000	>1000	130	100	70	50	20	10	>1000	900
3	A	900	900	300	300	50	20	20	10	>1000	1000
	B	500	500	20	20	10	0	2	1	1000	800
	C	300	100	100	70	0	0	0	0	300	0
	D	300	100	100	20	6	0	0	0	300	150
4	A	>1000	>1000	1000	300	900	700	1000	170	1000	700
	B	⩾1000	>1000	>1000	1000	>1000	300	>1000	1000	⩾1000	⩾1000
	C	50	20	170	100	30	2	170	130	150	100
	D	400	100	230	200	20	2	170	130	300	100
5	A	>1000	1000	1000	300	100	0	7	2	>1000	>1000
	B	900	900	170	100	30	1	0	0	900	700
	C	500	300	20	20	20	0	30	10	300	100
	D	700	500	130	100	6	4	10	3	700	500
6	A	⩾1000	⩾1000	230	200	40	5	1	0	⩾1000	⩾1000
	B	800	500	170	100	>1000	1000	100	50	>1000	1000
	C	400	0	70	30	50	3	7	0	500	0
	D	900	700	70	50	70	10	10	3	500	300

"Streptococci" estimated by inspection. A = first-drawn milk (50 ml.); B = next-drawn milk (50 ml.); C = mid-milk; D = strippings. > = greater than; ⩾ = much greater than. "Deposit" figures obtained by centrifuging 10 ml. milk and computing as for 1 ml. milk. The actual figures found were three times as high, since the deposit was taken up in 3 ml. of saline.

Table II. *Attempted enrichment of mastitis streptococci in milk samples*

Addition	Microscopic examination	Blood agar		Edwards's medium	
		Organisms other than streptococci	Streptococci	Organisms other than streptococci	Streptococci
(Control)	+ +	+	+ +	+	+
5% autolysed yeast	0	+ +	+ +	+ +	+
1% sucrose	0	+ +	+ +	+ +	+
2% serum	+	+ +	+ +	+ +	+
20% serum	+ + + +	+ +	+ +	+ +	+
5% blood	+	+ +	+ +	+ +	+
10% blood	+ + +	+ +	+	+ +	+
20% blood	+ + +	+ +	+ +	+ +	+ +
Crystal violet:					
1 in 400,000	0	+ +	+ +	+ +	+
1 in 200,000	0	+ +	+ +	+ +	+
1 in 100,000	Few	+ +	+	+ +	+

All samples incubated 24 hr. at 37° C.

TREATMENT OF SAMPLES

It is a common practice for producers to send samples of milk by post to laboratories for examination for mastitis streptococci. In our earlier experiments, samples were stored at room temperature overnight. Very high, and apparently erratic, counts were obtained, and, of course, less confidence was felt in the results of the other tests described in the second paper. Holding at room temperature (or at 37° C.) sometimes results in an overgrowth of mastitis streptococci by common lactic streptococci. In addition, as we were interested in aspects other than the presence of *Str. agalactiae* (e.g. total count, rennet coagulability, etc.) it was desired to obtain as accurate an assessment of the bacteriological and biochemical properties of the samples as possible.

Plating the samples immediately on returning to the laboratory (about 3 hr. after taking) was found to give much lower counts, clearer cut results and, of course, much greater reproducibility than when the samples were 16 hr. old. It is known that bacteria do not multiply appreciably in milk during the first few hours after milking. The results obtained were, for bacteriological data, very consistent. Thus, for example, no sample from cows free from mastitis gave a count on milk agar of over 500 per ml. Such a procedure gives as accurately as possible a measure of the udder flora of each cow. All the tests described in these papers were done within a few hours of taking the sample.

TECHNIQUE OF PLATING

In order to expedite the plating of large numbers of samples, the following technique was adopted:

Immediately on returning to the laboratory 1 ml. and 1 drop ($=\frac{1}{30}$ ml. approx.)[1] of the well-shaken milk samples were transferred to sterile Petri

[1] A better method is to add 1 and 0·1 ml. with a specially calibrated pipette.

dishes and 5% blood crystal violet aesculin agar (Edwards's medium) added and mixed in the usual way. Cows' blood was used throughout. After incubation for 24 hr. at 37° C. the plates were examined for haemolytic colonies. These were noted and the plates incubated for a further 24 hr. They were then examined in a suitable light and recorded. It was found useful to leave the plates for a further 2 days at room temperature, as, during this time, small colonies of staphylococci increase in size and opacity, whereas those of streptococci do not alter appreciably. Many "doubtful plates" thus cease to be doubtful. Samples were also plated in plain blood agar, milk agar, and sometimes in Edwards's medium without blood, starch agar and brom-cresol-purple aesculin agar.

Except in obvious cases, colonies suspected to be *Str. agalactiae* were picked off for confirmation. Colonies of *Staphylococcus* may be mistaken for *Str. agalactiae* in Edwards's medium, even forming zones of "weak β" haemolysis. These zones are, however, usually uniformly "weak β" (i.e. contain a uniform small concentration of corpuscles), whereas *Str. agalactiae* zones are clear near the colony and then become less clear (i.e. contain more corpuscles) as the distance from the colony increases. Although *Str. agalactiae* should theoretically give a light-coloured colony as it does not ferment aesculin, in practice all shades of colour from a pale slate to a deep grey are found, and cocci (*Staphylococcus*, *Micrococcus* and *Sarcina*) cannot always be differentiated by colour alone. Aesculin is an unstable sugar, especially at *p*H 7·4, so that decomposition during sterilization of the medium may be a factor in the formation of the dark zones around non-aesculin-fermenting colonies.

Choice of media

The medium devised by Edwards(8) was used as a basis for comparison in this work. This medium contains crystal violet (1 in 500,000) to inhibit organisms other than mastitis organisms. It is, therefore, most valuable for the examination of samples sent by post in which the miscellaneous flora has increased. Actually *Str. agalactiae* may be slightly inhibited (Table III) and staphylococci may be completely or almost completely inhibited. Where samples can be examined within a few hours of taking, it seems advantageous on the basis of our work to use a simple blood agar. This point is illustrated in Table I.

The ability of various species of streptococci to grow in Edwards's medium is obviously an important point. Experiments were therefore made to determine which types of streptococci could grow in this and other media. The results are given in Table III.

It will be seen that *Str. agalactiae* grows almost equally well in plain blood agar, Edwards's medium, and in the brom-cresol-purple aesculin agar. The starch agar proved to be a poor medium, presumably because of the low concentration of fermentable sugar (sucrose). The composition of these

Table III. *Growth of type cultures in various media*

Strain	Description	Plain blood agar: Count	Plain blood agar: Haemolysis type	Plain blood agar: Colour of colonies	Aesculin crystal violet blood agar, Edwards's medium: Count	Aesculin crystal violet blood agar, Edwards's medium: Haemolysis type	Aesculin crystal violet blood agar, Edwards's medium: Colour of colonies	Starch agar: Count	Starch agar: Colour of colonies	BCP-aesculin agar: Count	BCP-aesculin agar: Colour of medium	BCP-aesculin agar: Colour of colonies
I. Saprophytic streptococci												
Str. cremoris OJ	Stock	0	—	—	0	—	—	0	—	0	Purple	—
Str. lactis OJ	,,	200	—	—	0	—	—	200	—	150	Yellow	Yellow-brown
Str. faecalis 775	,,	1000	—	—	1000	—	—	1000	—	1000	Yellow-green	Deep yellow
Str. faecalis 2707	,,	300	sl., bl., gr.	Green	300	—	Black	300	Cream	300	,,	Yellow
Str. glycerinaceus 2702	,,	>1000	gr.	—	>1000	bl., gr.	Brownish	>1000	—	>1000	Red-brown	Dark brown
Str. bovis (?) 2701	,,	>1000	bl.	Green	>1000	sl., gr.	Green	⩾1000	—	⩾1000	Yellow-green	Deep yellow
Str. bovis L 5	Freshly isolated	0	—	—	0	—	—	0	—	0	Purple	—
Str. bovis Penelope	,, ,,	0	—	—	0	—	—	0	—	0	,,	—
Str. inulinaceus OJ	Stock	1000	α′	—	1000	gr.	Black	1000	—	1000	Yellow-green	Black
II. Pathogenic streptococci												
Str. pyogenes 2400	Stock	300	β (wide)	Black	0	—	—	0	—	300	Purple	Dark brown
Str. pyogenes Angel	,,	300	β (wide)	—	200	β	—	300	—	300	Blue	Black
Str. mitis (?) B 7	,,	100	bl., gr.	—	150	—	Black	70	Sl. cream	50	Yellow-green	Deep black
Str. mitis (?) B 10	,,	300	gr.	—	300	gr.	—	400	,,	300	,,	,,
Str. mastitidis 3481 (I)	,,	300	Weak β	—	300	Weak β	—	200	—	300	Purple	—
Str. mastitidis 3444 (I)	,,	15	Weak β	—	1	Weak β	—	0	—	50	,,	Brownish
Str. mastitidis OJ (I)	,,	300	—	—	300	—	Brownish	300	—	300	,,	—
Str. mastitidis 182 (I)	Freshly isolated	150	bl. or weak β	—	300	Weak β	—	0	—	70	,,	Yellow with yellow zones
Str. mastitidis 453 (I)	,, ,,	200	β (narrow)	—	200	β (narrow)	—	0	—	0	,,	—
Str. mastitidis 923 (II)	,, ,,	400	α	—	300	α	Black	0	—	800	,,	Black
Str. mastitidis Bulk (II)	,, ,,	400	Weak β	—	400	Weak β	—	400	Sl. cream	400	Yellow	Deep yellow and yellow-brown

(I) = Minett's group I; (II) = Minett's group II; sl. = slight; bl. = "bleaching"; gr. = "greening".

Starch agar = 500 ml. peptic casein digest (14), 1 g. sucrose, 10 g. starch, 50 ml. autolysed yeast (18) and 15 g. agar made up to 1000 ml.

BCP-aesculin agar = 500 ml. peptic casein digest, 10 g. sucrose, 1 g. aesculin, 0·1 g. $FeCl_3$, 100 ml. 0·04% brom-cresol-purple, 50 ml. autolysed yeast and 15 g. agar made up to 1000 ml. All media adjusted to *p*H 7·4.

Milk not added in these experiments.

media is given in Table III. *Str. cremoris*, *Str. lactis* and *Str. bovis* did not grow in Edwards's medium, the last failing to grow in any of the media. *Str. bovis* is perhaps of the greatest interest in this respect, since in culture it is most likely to be confused with *Str. agalactiae*. In our experience, *Str. bovis* grows with difficulty in most laboratory media, at any rate when first isolated, so that even if it were to find its way into samples taken under the conditions described, which we think unlikely, it would probably not form colonies.

Concentration of blood in blood agar

Experiments were made with various concentrations of blood in blood agar to ascertain whether certain concentrations would stimulate the growth and size of haemolytic zones of the mastitis streptococci and depress the growth of other organisms. The results are summarized in Table IV.

Table IV. *Order of superiority of media for revealing colonies and haemolytic zones of mastitis organisms and for suppressing "contaminants" in bulk milk*

	Dilution of bulk milk plated		
	1/10 ml.	1/100 ml.	1/1000 ml.
Farm 1	20%>10%>5%>CV	10%>20%=5%>CV	20%>10%=5%=CV
Farm 2	CV>5%	CV>5%	CV>5%
Farm 3	CV—no zones 5%—many staph. zones	CV—no zones 5%—some staph. zones	CV—no zones 5%—2 staph. zones

Percentages indicate concentration of blood in ordinary blood agar. >=superior to; CV = crystal violet aesculin blood agar.

It is evident that higher concentrations of blood may yield superior media from the point of view of number and size of colonies and distinctness of haemolytic zones, but involve obvious technical disadvantages (difficulty of mixing blood into medium and the larger quantity of blood required).

The advantages and disadvantages of blood in media for mastitis investigations

Many laboratories cannot obtain large quantities of sterile blood so that the value of blood in the medium is an important point. Blood agar has one important advantage, however, as those colonies surrounded by the "weak β" type of haemolysis characteristic of many strains of *Str. agalactiae* are immediately recognized. The disadvantages, as we see them, are as follows:

(1) The difficulty of obtaining blood.

(2) The fact that undue significance may be attached to haemolysis, since it is not correlated with pathogenicity or type.

(3) The impossibility of using hydrogen peroxide for the catalase test in blood media.

(4) The labour and time involved in preparing the blood media and testing the blood for sterility.

Further, it may be remarked that milk appears to be as good as blood or serum as nutritive material for mastitis streptococci. Thus, it has been frequently observed that blood agar plates containing 1 ml. of milk may be crowded with typical *Streptococcus* colonies when the $\frac{1}{30}$ ml. plate is sterile or contains only very few colonies. Plating the milk itself (instead of centrifuged deposits) will thus yield a richer nutritive medium but in many cases prevent the appearance of haemolytic zones on account of the acid formed from the lactose of the milk. Examination of plates after 24 hr. at 37° C. will frequently permit the detection of haemolysis in such cases.

MEDIA NOT CONTAINING BLOOD

A number of experiments was made with various media both to obtain a differential medium and the highest possible count in "udder samples". Milk appears to supply all the nutritive materials required, since additions of yeast, plant extracts and dextrose to milk do not significantly increase the growth rate of mastitis organisms (12). Further, the addition of 2% of 10% malt extract to milk agar was found to have no significant effect on the count. Media containing starch (which gives an orange pigment with many strains of mastitis streptococci) and sucrose (which is strongly fermented by these streptococci) together with an indicator did not give good differentiation. Ordinary milk agar (13) was found to give excellent growth of mastitis streptococci. It has the great advantage that it is simple to make and is in common use. Of various digest media (peptic, pancreatic and double-digests of casein and milk) a peptic milk digest has been found to give the best growth of *Str. agalactiae* (12). Peptonized milk agar (14) may therefore be considered as an alternative to milk agar, and perhaps preferable if centrifuged deposits are plated, as in this case milk is not added to the medium.

Such media are, however, not selective and therefore small numbers of streptococci in a fairly high total count may be missed.

CONCLUSIONS AS REGARDS MEDIA TO BE USED

It is suggested that if only the incidence of mastitis in a herd is under investigation, plain blood agar be used provided that the samples can be plated within a few hours of taking. Under these conditions, the use of an inhibiting agent (e.g. crystal violet) is unnecessary, and not only is a truer quantitative picture of the udder flora obtained but there will be no danger of missing types inhibited by crystal violet. Thus a case of subclinical mastitis caused by group III (Minett) was recently overlooked at first because the streptococci did not grow on Edwards's medium (15). Staphylococci are also frequently inhibited and *Corynebacterium pyogenes*, the cause of "summer mastitis" does not grow on Edwards's medium (16). If large numbers of staphylococci are present, the possibility of the presence of staphylococcal mastitis should be investigated (17). A further advantage, especially if the general

udder flora is a matter of interest, as it should be if first quality milk or milk products are to be produced, is that plain blood agar gives counts similar to those obtained with milk agar (Table I). If blood is unobtainable, milk agar or peptonized milk agar should be used. The advantages of these media may be summarized as follows:

(1) A measure of the "total udder count" is obtained.

(2) In the absence of inhibiting agents no types are suppressed (e.g. Minett's group III streptococci, staphylococci and *Corynebacterium pyogenes*).

Additional advantages of milk agar and peptonized milk agar

(3) Being simple media they are readily prepared, stored and used.

(4) Hydrogen peroxide may be used to differentiate streptococci (catalase-negative) from staphylococci and other organisms (catalase-positive).

When it is desired to pick off colonies for confirmation, hydrogen peroxide (*ca.* 3 vol.) should only be added to a portion of the plate and the agar pricked with a needle to bring the hydrogen peroxide into contact with the colonies.

On these non-blood media it is naturally essential to confirm by serological or cultural tests. For this purpose, if serological methods cannot be used, it is recommended to pick off at least two of the colonies most likely to be *Str. agalactiae* into yeast dextrose litmus milk. Acid or acid clot without reduction is sufficient to characterize the type for routine purposes. Reduction of the litmus may indicate Minett's group II or III (11), but more probably a harmless lactic acid *Streptococcus*. It is then essential to type the strain by means of the tests indicated (Table VI). Even when using the crystal violet medium, it is advisable to confirm all but the very obvious cases (i.e. large numbers of typical colonies causing characteristic "weak β" haemolysis).

CULTURAL CONFIRMATION OF SUSPECTED COLONIES

Various methods were tried with the object of obtaining the simplest and surest cultural tests for confirmation. A study of the cultural characteristics of the streptococci and of the milk flora in general showed that the most likely source of error would be to mistake *Str. bovis* for *Str. agalactiae*.

Starch broth was found to be quite useful, as in this medium *Str. agalactiae* produces no acid but frequently an orange pigment, and *Str. bovis* produces acid but no pigment. These reactions are, however, only 80–90% reliable, and ultimately the following procedure was adopted: The colony was picked off into yeast dextrose litmus milk (18) and incubated for 48 hr. at 37° C. Plain litmus milk sometimes failed to give growth. A further advantage of yeast is that some types, e.g. *Str. inulinaceus* and also attenuated strains of other normally strongly reducing streptococci which only reduce litmus slightly in plain litmus milk, will usually reduce strongly in the reinforced medium (18). Of all the streptococci which might be found in samples taken in this way, only *Str. agalactiae* and *Str. bovis* give an acid reaction, usually with clot, but fail to reduce litmus significantly in yeast dextrose litmus milk.

Cocci which are frequently mistaken for streptococci even on Edwards's medium, either produce no visible change in yeast dextrose litmus milk (non-dextrose fermenting) or produce acid, clot and almost complete reduction nearly to the surface (dextrose-fermenting). In cases of doubt 1 ml. of the culture may be added to a few ml. of *ca.* 3 vol. hydrogen peroxide. Cocci other than streptococci, containing catalase, produce bubbles of oxygen; streptococci which never contain catalase do not. In the present investigation the yeast dextrose litmus milk culture was further subcultured into hippurate broth and this medium tested for hydrolysis of hippurate to benzoic acid and glycine. Hydrolysis of hippurate is perhaps the most specific biochemical characteristic of *Str. agalactiae*. *Str. bovis* does not hydrolyse hippurate, at any rate strongly, so that this test readily differentiates the two types. In the hippurate test it is essential to titrate each new batch of hippurate broth with ferric chloride solution as follows, otherwise misleading results will be obtained:

To 2 ml. of uninoculated control (incubated 5 days at 37° C.) add drop by drop a 7 % solution of ferric chloride (prepared from commercial 58 % solution) by means of a calibrated 1 ml. pipette. At first a precipitate containing protein matter and hippuric acid is formed. On continued addition, this dissolves and the least amount of ferric chloride required to dissolve the precipitate is the amount to use for the test, i.e. to add to 2 ml. of the incubated culture.

If desired, the starch broth test may be used as an alternative or in addition to the hippurate test.

A number of strains was subjected to the biochemical tests used by Minett and his collaborators and summarized by Minett(11) (cf. Table VI). The findings of these workers were confirmed, it being possible to classify the strains studied in one of the three groups postulated, a few strains being slightly atypical (Table V). Since it has been found that some strains of group I are able to reduce methylene blue at a concentration of 1 in 20,000(19) a concentration of 1 in 15,000 was used with completely satisfactory results, all group I strains failing to reduce the dye at this concentration.

In Table VI are given the characteristics used by Minett(11) together with additional data upon mastitis and other streptococci likely to be found in milk (cf. also(20)).

NUMBER OF TESTS REQUIRED FOR DIAGNOSIS

It is well recognized that infected cows may excrete mastitis streptococci in their milk intermittently. It is thus possible that an infected cow may be tested many times before a positive result is obtained. In practice, however, it is impossible to make a long series of tests, and in the present work it was decided that each animal should be tested twice at intervals of not less than 10 days for diagnosis at any one time. It is realized that a few of the milder cases may be missed; a larger number of tests, while desirable for research purposes, is not an economic proposition for eradication purposes, to which end the present work was directed.

Table V. *Main characteristics of mastitis streptococci isolated from udder samples (random selection)*

Code no.	Microscopic appearance	Growth in yeast dextrose broth		Hydrolysis of sodium hippurate	Reduction of methylene blue (1 in 15,000)	Litmus milk	Group (Minett)
		Turbidity	Sediment				
14	ML	–	+ +	+ +	–	AV1″r	I
91	M			tr.	–	AV	,,
09	L	–	+ +	+ +	–	AV	,,
82	L	–	+ +	+ +	–	AV	,,
11	ML	–	+ +	+ +	–	AC¼″r	,,
44	PS	–	+	+ +	–	AC¼″r	,,
34	SL	+	+	+	–	A	,,
13	PS	–	tr.	+ +	–	AC	,,
52 (2)	L	–	+ +	+	–	AC	,,
89	L	–	+ +	+	–	AV1″r	,,
11	ML			+ +	–	A¼″r	,,
16	M	–	+ +	+ +	–	AC¼″r	,,
07	ML	–	+ +	+ +	–	AV	,,
53	S	–	+ +	+ +	–	AC mott.	,,
87	–	–	+ +		–	A	,,
63	–	–	+	+ +	–	AC	,,
96	–	–	+	+ +	–	AC1″r	,,
23	M	+	+	–	R	A¼″r	II
Bulk	–	+	+ +	–	R mott.	AC1″R	,,
52 (3)		+	tr.	tr.	R	AVR1″	,,

P = pairs; S = short chains; M = medium chains; L = long chains. No strain fermented mannitol.

In litmus milk: A = acid, C = clot, V = viscous, R = complete reduction and r = partial reduction to extent indicated; mott. = mottled (localized areas of reduction). tr. = trace.

Cultures incubated 5 days at 37° C.

Classification of results

In order to assess the severity of the mastitis or abnormality, the results of all tests were placed in four groups: 0, (+), + and + +. The standards adopted are given in Table I of the second paper in this series (p. 75 of this *Journal*).

Comparison of tests with "diagnosis"

In any series of tests of any cow (usually two tests were made) the grouping of the highest number of colonies of *Str. agalactiae* per ml. found in Edwards's medium has been taken as the "diagnosis" (e.g. three results such as 0, + + and (+) would give a "diagnosis" of + +). The results of individual tests were then compared with the "diagnosis," the results being given in Table VII. In these tests the milk was plated direct. The technique was thus slightly different from that of Minett *et al.* (3) (cf. Paper II, Table II).

Thus of a total of 124 tests made on animals known to be badly infected forty-two (34%) gave a negative result. Similarly, of sixty-four tests on animals moderately infected twenty-three (28%) gave a negative result, and of sixty-eight tests on animals lightly infected thirty-four (50%) gave a negative result.

Table VI. *Cultural characteristics of mastitis and other streptococci found in milk*

Yeast — Heat resistance

Blackening

Table VI. *Cultural characteristics of mastitis and other streptococci found in milk*

Type	Growth at 10° C.	Growth at 15° C.	Methylene blue milk 37° C.	Yeast dextrose litmus milk 37° C.	Catalase	Blackening with aesculin and $FeCl_3$	Growth in Edwards's medium	Hydrolysis of hippurate	Haemolysis type	Heat resistance 15 min. at 60° C.
Str. mastitidis (group I)	0	Slow	0	**AC**	**0**	**0**	**+**	**+**	**0 or β (weak)**	**0**
Str. mastitidis (group II)	0	Slow	(C) R	**ACR**	**0**	**0**	**+**	**0**	0 (G)	0
Str. mastitidis (group III)	+ ?	+	(C) R	**ACR**	**0**	**+**	**0**	**+**	0	0
Str. pyogenes (human)	0	0	0	AC	0	+	±	0	β	0
Str. pyogenes (bovine)	0	0	0	AC	0	0	+	0	β	0
Str. "viridans" (salivarius)	0	+	CR	ACR	0	±	+	0	α	∓
Str. bovis	0	0	(C)(R)	AC	0	+	0	0*	0 (G)	+
Str. inulinaceus	+	+	(R)	AC	0	+	+	0	0 (G)	+
Str. thermophilus	0	0	0	AC	0	0	0	0*	0 (G)	+
Str. cremoris	+	+	CR	ACR	0	±	0	0	—	+
Str. lactis	+	+	CR	ACR	0	+	0	±	0 (G)	+
Str. faecalis	+	+	CR	ACR	0	+	+	∓	0 (G)	+
Str. glycerinaceus	+	+	CR	ACR	0	+	+	∓	0 (G)	+
Str. liquefaciens	+	+	CR	ACR	0	+	+	∓	0 (G)	+
Udder cocci (dextrose-fermenting)				**ACr**	**+**		**+**			
Udder cocci (non-dextrose-fermenting)				**0**	**+**		**+**			

Type	Mannitol	Sorbitol	Trehalose	Raffinose	Salicin	Aesculin: Acid	Aesculin: Blackening with $FeCl_3$	Starch	Inulin
Str. mastitidis (group I)	0	0	+	0	±	0	0	0†	0
Str. mastitidis (group II)	0	0	∓	0	0	0	0	0	0
Str. mastitidis (group III)	+	0	0	0	+	+	+	+	+
Str. pyogenes (human)	∓	0	+	0	±	±	±	0	0
Str. pyogenes (bovine)	0	+	0	0	+	0	0	+	0
Str. "viridans" (salivarius)	0	0	±	±	+	±	±	0	0
Str. bovis	0	0	±	+	+	+	+	+	0
Str. inulinaceus	∓	0	±	+	+	+	+	0	+
Str. thermophilus	0	0	0	0	0	0	0	0	0
Str. cremoris	0	0	0	0	0	±	±	0	0
Str. lactis	∓	0	+	0	+	+	+	0	0
Str. faecalis	+	±	+	0	+	+	+	0	0
Str. glycerinaceus	+	+	+	∓	+	+	+	0	0
Str. liquefaciens	+	+	+	∓	+	+	+	0	0

A = acid; C = clot; R = complete reduction almost to surface; r = partial reduction almost to surface; G = "greening" or discolorati on; * = occasional exceptions; † = orange pigment usually found at bottom of tube; ± = usually fermented; ∓ = usually not fermented.

The characteristics on which our method of differentiation is based are printed in heavy type.

Table VII. 256 *tests*

Results of single tests

"Diagnosis*"	++	+	(+)	0	Total
++	70	9	3	42	124
+	0	34	7	23	64
(+)	0	0	34	34	68
					256

* Only infected cows considered.

Errors in "diagnosis" due to the limited number of tests employed

In this series of double tests (at 10-day intervals) the following results were obtained:

Result	No. of results
0 and 0	138
0 „ (+) and vice versa	35
(+) „ (+)	0
+ „ (+) „	7
+ „ 0 „	51
+ „ +	25
Total	256

0 =no colonies of mastitis streptococci found;
(+)=1 to 10 colonies of mastitis streptococci per ml. found;
+ =>10 colonies of mastitis streptococci per ml. found.
(++ not used here.)

It may be observed that of forty-two results containing a (+), thirty-five were accompanied by a 0, seven by a + and none by a second (+).

In view of the claim by Hucker(21) that the udders of virgin heifers may contain streptococci resembling *Str. agalactiae* and of the possibilities of contamination in the taking of samples (a possibility that cannot be ruled out even with the most careful technique), and finally of the organism existing in the teat canal in the passive (carrier) condition, the significance of small numbers of organisms in udders otherwise yielding apparently normal milk remains an open question. Nevertheless, from the point of view of eradication and control, such cows were regarded as positive and placed in the "infected" herd(7).

Costs of examination. In practice the cost of a remedy or precaution, and its probable saving are important factors. The latter point is discussed elsewhere(22). The cost of examination of a herd of fifty cows on two occasions has been estimated to be £6 0*s.* 0*d.* or about 2*s.* 6*d.* per animal. Assuming an average yield of 800 gal., an incidence of 40% and an average loss of 15% of milk yield, the estimated loss at 1*s.* per gallon is about £120 in milk alone.

REFERENCES

(1) MUNCH-PETERSEN (1938). "Bovine mastitis." *Imp. Bur. Anim. Hlth Rev. Ser.* no. 1.
(2) STABLEFORTH (1930). *J. comp. Path.* **43**, 22.
(3) MINETT *et al.* (1930). *J. comp. Path.* **43**, 163.
(4) MINETT & STABLEFORTH (1931). *J. comp. Path.* **44**, 114.
(5) MINETT *et al.* (1932). *J. comp. Path.* **45**, 1.
(6) EDWARDS (1932). *J. comp. Path.* **45**, 43.
(7) MINETT *et al.* (1933). *J. comp. Path.* **46**, 131.
(8) EDWARDS (1933). *J. comp. Path.* **46**, 211.
(9) —— (1934). *J. comp. Path.* **47**, 49.
(10) STABLEFORTH *et al.* (1935). *J. comp. Path.* **48**, 300.
(11) MINETT (1936). *J. Hyg., Camb.*, **35**, 504.
(12) DAVIS (1936). Unpublished observations.
(13) HISCOX *et al.* (1932). *J. Dairy Res.* **4**, 105.
(14) DAVIS (1939). *J. Dairy Res.* (in the Press).
(15) MATTICK & SHATTOCK (1938). Unpublished N.I.R.D.
(16) ZEIN-EL-DINE, Reading University (1938). Private communication.
(17) MINETT (1937). *J. comp. Path.* **50**, 101.
(18) DAVIS (1935). *J. Dairy Res.* **6**, 121.
(19) CUNNINGHAM (1936). "The occurrence and detection of streptococcal mastitis." *Proc. Soc. Agric. Bact.*
(20) DAVIS (1939). In preparation.
(21) HUCKER (1937). *Tech. Bull. N.Y. agric. Exp. Sta.* no. 241.
(22) DAVIS (1938). *Dairy Industr.* **3**, 409.

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Note added 6 February 1939 (ref. p. 68). Dr P. M. F. Shattock and Dr A. W. Stableforth have drawn our attention to the fact that some strains of haemolytic streptococci (Groups A, C and E) may clot litmus or yeast dextrose litmus milk in 24 hours with only slight reduction. In all doubtful cases, and when non-blood media are used, both confirmatory tests should be carried out. This double confirmatory test (i.e. yeast dextrose litmus milk *and* hippurate broth) is very reliable.

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210. STUDIES IN MASTITIS

II. A COMPARISON OF METHODS FOR THE DETECTION OF SUBCLINICAL MASTITIS

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Many tests have been used for the detection of subclinical mastitis and these have been enumerated by Munch-Petersen(1) and Rosell(2). In view of the continued recommendation and use of these tests in many countries it was felt desirable to make a careful assessment of their value.

Using the results obtained by plating the milk twice in Edwards's medium as a criterion, the following were compared:

(1) Plating the deposit from 10 ml. milk in the same medium.
(2) Direct plating of milk in Edwards's medium without blood.
(3) Direct plating of milk in blood agar.
(4) Direct plating of milk in milk agar.
(5) Methylene blue reduction test.
(6) Catalase test.
(7) Rennet test.
(8) "Growth of starter" test.
(9) Brom-cresol-purple paper test.
(10) Incubation test (with addition of litmus solution).
(11) Titratable acidity test.
(12) Clinical examination.

A considerable number of results was obtained with the first nine tests which will now be discussed in detail.

Classification of results

In order to compare the results of the various tests and to ascertain the significance of degree of infection and extent of abnormality, the following arbitrarily selected groupings were chosen as the result of experience gained in the earlier experiments (Table I). These groupings are used throughout this series of papers.

(1) *Direct plating compared with plating of the deposit from* 10 *ml. milk.*

It will be obvious that plating the deposit from 10 ml. milk will be more likely to detect small numbers of mastitis streptococci than plating 1 ml. of the milk itself. Factors likely to reduce the numbers of colonies obtained from the deposit are (1) the fact that some of the organisms will be retained in the cream or not come down into the deposit, and (2) the small amount of milk

added to the medium (see p. 67) (cf. also Table I, Paper I). A number of experiments designed to compare these two methods gave the results shown in Table II. Both the milk and the deposit were plated in Edwards's medium.

Table I

Test	Grouping (degree of infection or abnormality) 0	(+)	+	++
Edwards's medium—colonies of mastitis streptococci per ml.	0	1–10	11–200	>200
Edwards's medium—total count per ml.	0–10	11–100	101–500	>500
Milk agar—total colony count per ml.	0–500	501–1000	1000–2000	>2000
Milk agar—*Streptococcus* colonies per ml.	0–50	51–100	101–500	>500
Methylene blue reduction time (hr.)	>12	9–12	3–9	<3
Catalase (height of oxygen in standard tube) (in.)	0–1¼	1½–1¾	2–2¼	>2¼
Rennet (time to clot in minutes)	20–30	31–60	61–180	>180
Growth of starter		Individual assessment		
Incubation test (number of days without visible change in litmus milk at 37° C.)	>3	3	2	1
Brom-cresol-purple (colour)	Dove grey	Bluish grey	Purple grey	Purple

Table II

Centrifuged deposit from 10 ml. milk (single test)

Direct plating of 1 ml. milk (single test)	++	+	(+)	0	Total
++	8	0	0	3	11
+	1	7	2	0	10
(+)	0	0	3	4	7
0	0	4	15	40	59
					87

Thus of forty-seven samples found to be positive (++, + or (+)) by one or both tests, seven were detected by direct plating but missed by the deposit method, and nineteen found by the deposit method but missed by direct plating. As might be expected, plating the centrifuged deposit reveals a somewhat higher number of "lightly infected" cows, but even this method does not reveal all cases by a single test.

The relative ease and simplicity of direct plating introduces the question as to whether it is advantageous to use the time involved in centrifuging in making an additional test. The choice may depend upon circumstances such as laboratory equipment and ease of procuring samples.

(2) *Standard technique compared with plating on crystal violet aesculin agar (i.e. Edwards's medium without blood).*

An investigation of the suitability of crystal violet aesculin agar without blood was made. Samples were plated in this medium as well as in the normal medium with blood, and both plates examined by inspection and counted. The non-blood plate was then flooded with a solution of 1% ferric chloride

to allow those types fermenting aesculin to form the characteristic black pigment. Hydrogen peroxide was then added to pick out catalase-positive colonies. Using this technique, not only was excellent differentiation between aesculin-fermenters and non-aesculin-fermenters obtained, but the addition of ferric chloride catalysed the decomposition of hydrogen peroxide by the weaker catalase-producing cocci. Sometimes colonies passed for streptococci when treated with hydrogen peroxide alone, but were readily detected as catalase producers when ferric chloride was added. Colonies of this type growing in a blood medium may pass for streptococci, and their real nature may not be proved until the confirmatory test in yeast dextrose litmus milk has been made (see first paper). Thus blood permits diagnosis by inspection with the haemolytic types (roughly 50% of the total positives in our experiments) but prevents the use of hydrogen peroxide. Since *all* streptococci are catalase-negative, and only about half the strains of *Str. agalactiae* produce the typical "weak β" type of haemolysis, it is evident that the catalase test with hydrogen peroxide is comparable in value with the "haemolysis test". When care was taken to confirm the nature of the suspected organisms, little difference could be detected between these two media.

(3) *Standard technique compared with plating on (plain) blood agar.*

The presence of crystal violet in Edwards's medium has the following effects:

(*a*) It considerably depresses most organisms other than mastitis streptococci. This partial inhibition may sometimes result in colonies of cocci simulating those of streptococci.

(*b*) It may slightly depress the growth of mastitis streptococci (Groups I and II—Minett).

(*c*) It has sometimes been found to inhibit some types of organisms causing a mastitis, which although usually described as being "acute" in nature, may nevertheless sometimes not be suspected (those caused by staphylococci, Minett's Group III *Streptococcus* and *Corynebacterium pyogenes*). The use of crystal violet, while excellent for its purpose, may thus result in failure to detect cases of mastitis other than those due to *Str. agalactiae* which would otherwise readily be detected.

(*d*) It may influence the type and extent of "haemolysis" produced.

(*e*) It does not inhibit a coccus (*Sarcina*) that causes "greening" on blood media and is frequently present in "udder samples". This organism has also been found and described by Minett (3).

A series of tests was made to compare the results obtained with Edwards's medium with those given by plain blood agar. As far as *Str. agalactiae* was concerned excellent agreement was usually found although naturally much higher total counts were obtained in the simpler medium.

(4) *Standard technique compared with (a) total count on milk agar, (b) streptococci on milk agar,* and

(5) *Standard technique compared with methylene blue reduction time.*

These data are given in the third and fourth papers of this series.

(6) *Standard technique* v. *catalase test.*

The catalase test is one of the oldest and best known of the indirect tests for mastitis. It is a measure of the cell content of the milk; except in rare instances the bacteria do not play a significant rôle in the production of oxygen from the added hydrogen peroxide.

Method. A very simple apparatus was devised as follows: Standard calibrated test tubes 6 × 11/16 in. were fitted with rubber bungs bored with a red-hot knitting needle to give a fine hole. One ml. of 20 vol. hydrogen peroxide was added to the tube and the milk sample added to fill the tube, the bung being inserted so that no air space remained. The tube was then inverted and held at room temperature (15–18° C.) overnight from about 7 p.m. until 9–10 a.m. the next morning. The height of the gas column was then measured with a ruler. This test is not only very simple but has the additional advantage that the result is not significantly affected by small changes in conditions, e.g. time of the test, concentration of hydrogen peroxide, etc.

The results of the catalase tests compared with the standard test for diagnosis are given in Table III.

Table III

"Diagnosis"	Catalase test ++	+	(+)	0	Total
++	20 (7)	6 (3)	13 (2)	32 (8)	71 (20)
+	7 (3)	3 (0)	9 (4)	15 (8)	34 (15)
(+)	4 (0)	1 (3)	9 (2)	15 (5)	29 (10)
0	16 (8)	15 (5)	16 (4)	91 (16)	138 (33)
					272 (78)
					350

The figures in brackets give the results for cows in late lactation. A cow was considered to be in late lactation when it had been more than 8 months in milk *or* was giving a yield which had decreased to less than 1 gal. per day. The stage of lactation has some effect upon the catalase test.

It will be seen that of 171 tests on presumably negative cows, forty-four (26%) gave a positive catalase reaction. Further, of ninety-one severely infected animals forty (44%) gave a normal catalase reaction, of forty-nine moderately infected cows twenty-three (47%) were catalase negative, and of thirty-nine lightly infected cows twenty (51%) passed the catalase test. It

would appear therefore, that the catalase test is of little value in the detection of mastitis.

(7) *Standard technique compared with the rennet test*, and

(8) *Standard technique compared with the "growth of starter" test.*

These tests are discussed in the fifth paper of this series.

(9) *Standard technique compared with the brom-cresol-purple paper test.*

This test, at once the simplest and one of the oldest tests for mastitis, is more commonly used in this country than any other. The observations were made on fore-milk (i.e. first-drawn milk) immediately before milking and the results are summarized in Table IV.

Table IV

Brom-cresol-purple test

"Diagnosis"	++	+	(+)	0	Total
++	10 (4)	21 (8)	8 (2)	45 (3)	84 (17)
+	5 (0)	15 (3)	5 (5)	13 (6)	38 (14)
(+)	1 (2)	7 (2)	11 (2)	30 (5)	49 (11)
0	3 (4)	25 (13)	17 (12)	143 (11)	188 (40)
					359 (82)
					441

The figures in brackets give the values for cows in late lactation.

It will thus be seen that of 188 tests on cows in full milk presumed free from mastitis twenty-eight (15%) gave a positive brom-cresol-purple test. Of eighty-four tests on badly infected cows forty-five (54%) gave a negative test, of thirty-eight tests on moderately infected animals thirteen (34%) reacted negatively, and of forty-nine tests on lightly infected cows thirty (61%) gave no indication of abnormality.

No cases of mastitis resulting in acid milk were found. Recently calved cows could be detected by the greenish grey colour given in the test.

The brom-cresol-purple test, like the catalase test, only detects about half the number of infected animals. It gives, however, fewer false positives than the catalase test.

Many workers appear to prefer brom-thymol-blue to brom-cresol-purple. Theoretically, and ignoring the colour effect of the milk itself, brom-thymol-blue should be a more sensitive indicator in the *p*H range of 6·6–7·2 (the average *p*H of milk is 6·65) since its *pK* value is about 6·8 and that of brom-cresol-purple somewhat lower, about 6·4. We prefer brom-cresol-purple,

however, not only in the paper test, but also in milk. The following factors may affect the choice of indicator:

(1) Personal preference and colour sense of observer.

(2) Concentration of dye in paper.

(3) Purity of sample of dye used.

The choice may thus be left to the individual worker.

(10) *Standard technique compared with the incubation test.*

Method. Single quarter samples of about 10 ml. were taken direct from the udder (as already described) into large test tubes (plugged and sterilized) containing 1 ml. of sterile 0·1% litmus solution(4). The tubes were then incubated at 37° C. for a few days, records of the changes being made each day. The test is thus a rough measure of the content of bacteria which can cause changes in litmus milk at 37° C. Samples from uninfected quarters nearly always withstand 3 days' incubation at 37° (i.e. show no change in the litmus or in the milk). Milk from quarters which were badly infected but showed no clinical symptoms usually showed marked changes in 1 day. This test was found to be about as good as any of the other indirect tests, but is naturally too empirical to be used quantitatively. Like the other tests it may be used for a preliminary "sorting out" of a herd. Where plating is impracticable, this test, combined with microscopic examination of the incubated samples, may be of considerable use.

(11) *Standard technique compared with titratable acidity determinations of quarter samples.*

Samples from individual quarters were titrated with *N*/9 NaOH within 3 hr. of milking. Badly infected quarters were readily detected, but the results were too irregular to enable any real significance to be attached to them.

(12) *Standard technique compared with clinical examination.*

About 10% of the cases of mastitis found by plating were detected by clinical examination by a skilled person. This proportion of cases, equivalent to an incidence of about 5% of the herd, is commonly taken as the true figure by farmers and others concerned with the economic aspects of the disease. It will be obvious that quite a false conception of the incidence of mastitis will be obtained in this way.

Other indirect tests

The following methods have been used for the detection of mastitis by various workers but were not tried extensively in the present investigation: the microscopic examination of incubated samples, the casein number determination, the leucocyte count, the sediment test, the curd tension test, the chloride test, the taste test, the electrical conductivity test, the lactose test and chlorine-lactose test.

An examination of the literature reveals that these tests, like those used by us, permit the detection of a variable proportion of infected cases. The first two (microscopic examination of incubated samples and the casein number determination, which has not yet been extensively investigated) appear to be

the most efficient (5, 6). It may be noted that similar conclusions on the value of the indirect tests have been reached by Rowland & Zein-El-Dine (7). Other tests have been described in the literature but need not be considered seriously.

The value of combined tests

The correlation between "diagnosis" and the combined results of the catalase, rennet and brom-cresol-purple paper test. Although the simple tests alone have been found to be of little use in detecting mastitis, there is the possibility that a combination of two or more of these might prove to be of some value. In Table V are given the data for the correlation between diagnosis on Edwards's medium and the result when the catalase, rennet and brom-cresol-purple paper tests are carried out and the highest result taken (e.g. catalase 0, rennet + and brom-cresol-purple (+) would be taken as +).

Table V

Combined result of catalase, rennet and brom-cresol-purple tests

"Diagnosis"	++	+	(+)	0	Total
++	18	10	6	8	42
+	8	8	4	3	23
(+)	3	8	8	9	28
0	18	24	18	46	106
					199

Table V shows, as might be expected, a lower proportion of missed positives but a much higher proportion of false positives. The unavoidable presence of false positives in these tests, therefore, constitutes an effective answer to those who continue to use them for eradication purposes. For this same reason, it is unlikely that any combination of these simple methods would furnish a satisfactory method of diagnosis for eradication purposes.

REFERENCES

(1) Munch-Petersen (1938). "Bovine mastitis." *Imp. Bur. Anim. Hlth Rev. Ser.* no. 1.
(2) Rosell (1933) "La mammite streptococcique de la vache." Quebec: Ministry of Agriculture.
(3) Minett (1937). *J. comp. Path.* **50**, 101.
(4) Davis (1935). *J. Dairy Res.* **6**, 121.
(5) Plastridge *et al.* (1935). *J. Dairy Sci.* **18**, 583.
(6) Rowland & Zein-El-Dine (1938). *J. Dairy Res.* **9**, 174.
(7) —— —— (1939). *J. Dairy Res.* **10**, 108.

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211. STUDIES IN MASTITIS

III. MASTITIS IN RELATION TO "UDDER COUNTS"

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By the term "udder count" in this series of papers is understood the colony count obtained when samples of early mid-milk (taken after about ½ pint has been removed from each quarter) are plated in milk agar and incubated for 2 days at 37° C. Dairy bacteriologists have long recognized the fact that cows with udder disease give high-count milk. In fact, some of those engaged in control work have made a practice of advising producers to search for mastitis when, in the absence of other causes, unusually high counts have been obtained in a series of samples. Therefore in low-count milk, at least, the milk agar plate gives some idea of infection in herds. The early observations, however, were not as a rule confirmed by satisfactory evidence of mastitis, i.e. by plating on blood agar or by cultural or serological typing of colonies picked off plates. This criticism also applies to many papers and statements in articles on mastitis as it affects other aspects of the dairy industry, e.g. off-taints, "slow starter" in cheese-making, and butter-making generally. Unless satisfactory evidence of mastitis is produced such work is valueless. Conclusions based on the results of the indirect tests are unreliable.

One of us (J. McC.) has, over a period of 8 years, observed that udder samples from cows suspected to be suffering from mastitis had higher counts than those from normal cows.

Prucha(1), Thornton & Strynadka(2), Johns(3), Johns & Hastings(4) and Pijanowski *et al.*(5) studied the relation between udder flora and mastitis. They found that diseased udders usually yielded milk of higher count than that from healthy udders.

In view of the importance of the relation between udder count and mastitis, both from the point of view of milk quality and of its possible usefulness as a means of diagnosis, a systematic investigation of the subject was made. Samples were plated simultaneously in Edwards's medium and in milk agar.

Standard technique (mastitis streptococci in Edwards's medium) compared with total count on milk agar

The correlation between the numbers of mastitis streptococci present and udder count was studied in various ways, e.g. by comparing the result of a single test in Edwards's medium against the result of a single test in milk agar, the diagnosis (highest result from two tests) against the result of a single test, etc.

Of all the so-called indirect tests the milk agar count was found to be the best, yielding very few false positives. Comparisons of single tests are shown in Table I.

Table I

Milk agar count (single test)

Edwards's medium (single test)	++	+	(+)	0	Total
++	37	11	7	13	68
+	4	0	3	36	43
(+)	5	0	3	41	49
0	7	11	11	374	403
					563

See Table I (p. 75 of this *Journal*) for explanation of groupings.

A more satisfactory picture is obtained when the diagnosis on Edwards's medium is compared with the milk agar count (Table II).

Table II

Milk agar count (single test)

"Diagnosis"	++	+	(+)	0	Total
++	41	11	7	55	114
+	10	1	3	42	56
(+)	1	0	4	61	66
0	0	5	4	243	252
					488

It may be seen that, of 170 moderately and badly infected cows, ninety-seven (57 %) failed to reveal any abnormality in udder count when one test only was made. On the other hand, no negative cow gave a really high count (> 2000) and only five of 252 (2 %) had a count of 1000–2000.

A still higher correlation was found between the diagnosis on Edwards's medium and the result when the higher of two udder counts was considered (Table III).

Table III

Milk agar count (higher of two counts)

"Diagnosis"	++	+	(+)	0	Total
++	28	6	4	9	47
+	5	1	1	15	22
(+)	1	0	3	27	31
0	0	2	3	107	112
					212

It will at once be evident that by using the result of two tests on milk agar (thus bringing the test into line with the diagnosis in Edwards's medium) a considerable improvement is effected. Thus of sixty-nine moderately and severely infected animals only twenty-four (35%) gave a low udder count (<500). Not one cow out of 112 free from mastitis gave a count of over 2000 and only two (2%) gave counts between 1000 and 2000. The evidence strongly suggests that by using three or more tests a still better correlation would be obtained since the false positives do not increase (a most striking fact) and the percentage of "misses" has fallen from fifty-seven (one test) (Table II) to thirty-five (two tests). If three or more tests in Edwards's medium had been made, the two "false positives" might well have become true positives.

THE CORRELATION BETWEEN TOTAL COUNT AND THE CATALASE FIGURE

Apart from the large number of samples giving low counts and low catalase figures, the correlation between these two properties was poor. The data are given in Table IV.

Table IV

Catalase figure

Milk agar count	++	+	(+)	0	Total
++	14	6	7	14	41
+	4	4	3	7	18
(+)	5	2	2	6	15
0	42	25	47	160	274
					348

In addition to estimating total counts on milk agar, the number of "streptococci" was also estimated. By this term is understood small, translucent, colourless colonies containing no catalase. The probability that other types might simulate streptococci in colony form in these circumstances is very small. The only likely types are those cocci growing slowly in milk agar. These are readily differentiated by the catalase test.

In Table V are shown the results when a single test on Edwards's medium is compared with a single estimation of "streptococci" on milk agar.

Table V

Milk agar "streptococci" (single test)

Edwards's medium (single test)	++	+	(+)	0	Total
++	42	10	1	2	55
+	5	8	8	15	36
(+)	3	13	2	28	46
0	14	42	35	291	382
					519

In Table VI are given the results when the diagnosis on Edwards's medium is compared with two tests estimating "streptococci" on milk agar (i.e. the higher result from two tests).

Table VI

Milk agar "streptococci" (two tests)

"Diagnosis"	++	+	(+)	0	Total
++	28	5	1	2	36
+	2	6	3	4	15
(+)	3	8	1	21	33
0	0	10	10	71	91
					175

From inspection of Tables V and VI it will be seen that seventeen of ninety-one (19%) and six of fifty-one (12%) moderately and severely infected cows were missed by this test. Only ten of ninety-one (11%) presumed mastitis-free cows were found to be positive (Table VI). In the milk agar plate, therefore, the total count gives a negligible number of false positives but misses about a third of the infected cows, while the "streptococci" count gives more false positives but misses only about 12% of the infected cows.

The possibilities of combining the data were, therefore, explored.

In Table VII are given the results when the diagnosis was correlated with total count *and* "streptococci" (one test only). The higher result was taken as the resultant, e.g. + (total count) and 0 (streptococci) = +.

Table VII

Total count and "streptococci" (one test)

"Diagnosis"	++	+	(+)	0	Total
++	48	15	11	19	93
+	7	11	8	23	49
(+)	3	11	6	43	63
0	2	17	199	180	398
					603

The results obtained when a combined "diagnosis" of count and "streptococci" (i.e. the highest of two tests or four results) is compared with the standard technique, are given in Table VIII.

Table VIII. *Standard technique* v. *"diagnosis" by total count and "streptococci" (highest of four results in two tests)*

"Diagnosis"	Milk agar count + "streptococci"				
	++	+	(+)	0	Total
++	29	5	1	2	37
+	2	5	3	4	14
(+)	3	7	3	19	32
0	2	10	9	71	92
					175

It will be seen that when only one test on milk agar is used, forty-two of 143 infected and heavily infected cows (29%) are missed, and of 218 mastitis-free cows, nineteen, or 9%, are falsely reported as positive. When two platings on milk agar are considered together, however, six of fifty-one moderately and badly infected cows (12%) are missed, and of ninety-two uninfected cows, twelve (or 13%) are wrongly reported as positive.

Consideration of these data shows that the ordinary milk agar plate affords a valuable means for diagnosing mastitis. A count of over 2000 may safely be taken as an indication of mastitis, while a count between 500 and 2000 suggests the probability that mastitis is present. A count of under 500, however, does not mean the absence of mastitis, as roughly one-third of the infected cows yield milk having a low count. If both total count and streptococci are considered, a small proportion of infected cows is missed and a small proportion of presumed mastitis-free cows is wrongly reported as positive.

In view of the fact that the groupings for "streptococci" in milk agar are different from those of mastitis streptococci in Edwards's medium (Table I, Paper II) there would appear to be little doubt that cultural confirmation of suspected colonies in milk agar would reduce both the missed cases of infection and the "false positives" to negligible proportions.

For eradication schemes, therefore, any medium such as milk agar, which gives good growth of mastitis streptococci, may be used provided that cultural or serological confirmation (such as that described in Paper I) is carried out, and the samples are taken "aseptically" and plated within a few hours of taking. Otherwise a selective medium such as Edwards's crystal violet blood agar must be used.

THE PRACTICAL IMPLICATION OF THE RELATION BETWEEN UDDER COUNT AND MASTITIS

In view of the fact that many infected cows give high-count milk (>2000 per ml.) and practically all mastitis-free cows give low-count milk (<500 per ml.) (see Table II) the data were scrutinized to see if any limit of count

could be accepted as indicating either definite infection or definite freedom from infection. It was found in this way that only on rare occasions did a sample with a count of <100 come from an infected cow. Thus of sixty-two samples with counts of less than 100 per ml. selected at random from our data, only one was from a cow infected with mastitis. This infection was very light, and, on a second testing, the sample gave a count of >100. A few of these samples were from cows previously found to be positive but apparently recovered, and, of course, from cows which subsequently contracted the disease. With the single exception, every cow was found by a double testing in Edwards's medium to be free from mastitis. When it is realized that even the standard technique does not reveal 100% of cases it may safely be asserted that, even for eradication schemes, a count of <100 may be taken as a safe indication of the absence of mastitis. A sterile plate or a very low count, however, should be regarded with suspicion, as it *may* indicate a severe mastitis (or injury) resulting in the presence of blood or plasma in the milk. Such samples, however, did not yield streptococci in the blood plate.

Of the other aspect, it will be seen from Table II that very few non-infected cows gave milk with a count of >500 (5%) and none at all with a count of >2000. It is, of course, possible if not probable, that further examination of this 5% would have revealed a very light infection. As a practical limit, a count of 1000 may be taken as an indication of mastitis.

Eradication scheme based on udder counts

As a procedure preliminary to the laborious examination of all cows in a herd, or where detailed bacteriological work is impracticable, the following may be recommended:

The fore milk is first tested at the farm by the strip cup and brom-cresol-purple paper tests. Cows reacting positive to both tests are recorded as *Positives* (6). Of the remaining cows, udder samples are taken as described and plated in milk agar or in blood agar. Cows giving samples with an udder count of less than 100 are recorded as *Negatives* and if giving a count of over 1000 as *Positives*. The remaining plates (i.e. those having a count between 100 and 1000) are then further examined by picking off likely colonies and confirming as described in Paper I. No cow should be pronounced negative unless at least two tests are made.

Edwards (6) found that about 30% of *infected* quarters could be detected by the strip cup test, and we have found that about a third of the *total* number of cows can be diagnosed as *Negatives* or *Positives* on the basis of udder count.

By this procedure, therefore, the number of cows to be tested by the full and rather involved technique is reduced by about half. When proper diagnosis is impossible, a count of over 500 could be taken, provisionally, to indicate mastitis.

REFERENCES

(1) PRUCHA (1933). *Rep. int. Ass. Dairy Insp., Wash.*, **22**, 81.
(2) THORNTON & STRYNADKA (1935). *Rep. int. Ass. Dairy Insp., Wash.*, 178.
(3) JOHNS (1936). *Ann. Proc. Int. Ass. Milk San.* p. 145.
(4) JOHNS & HASTINGS (1938). *Can. J. Res.* **16**, D, 15.
(5) PIJANOWSKI *et al.* (1937). *Pol. Agr. For. Ann.* **38**, z. 1.
(6) EDWARDS (1934). *J. comp. Path.* **47**, 49.

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212. STUDIES IN MASTITIS

IV. MASTITIS IN RELATION TO THE METHYLENE BLUE REDUCTION TEST

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(With 1 Figure)

The adoption of the methylene blue test as an official method of grading milk in England makes this subject one of considerable importance. The predominating factor in this, as in other tests, is the condition of storage (time and temperature) of the sample. If mastitis affects the methylene blue reduction time of milk the factors responsible may be any or all of the following:

(1) The mastitis organisms themselves.
(2) Other organisms present (secondary invaders).
(3) Body cells, e.g. the so-called leucocytes of milk and also blood cells.
(4) Chemical changes in the milk as the result of the mastitis.

A related aspect is the value of the methylene blue test on single cow or quarter samples as a test for mastitis.

(1) The reducing power of mastitis streptococci

A systematic investigation of a number of strains of *Str. agalactiae* in milk containing varying concentrations of methylene blue was made. The results are given in Table I and include the data obtained with a few strains of Groups II and III mastitis streptococci (Minett) and some poorly reducing streptococci. These results confirm previous findings and those of Edwards (1, 2) that *Str. agalactiae* either does not reduce methylene blue or does so with difficulty. It is thus unlikely that under practical conditions *Str. agalactiae* is a considerable factor in the reduction of the dye in the standard test. The shorter reduction times given with many samples of mastitis milk (Table IV) may be attributed to factors other than the activity of *Str. agalactiae*.

The numbers of *Str. agalactiae* present in raw milk as sold to the public will naturally vary, not only according to the extent of infection, but also depending on the treatment, age and temperature of holding of the milk. It is possible for milk to contain thousands of *Str. agalactiae* per ml. (colony count) without the state of affairs being suspected by the producer who makes no examination for the presence of mastitis.

Table I. *Effect of different concentrations of methylene blue on the reducing and milk clotting power of strains of* Str. agalactiae *and other streptococci*

Strain	Grouping (Minett)	Concentration of methylene blue—1 part in 1,000,000		500,000		250,000		100,000		50,000		20,000		10,000		Without dye
S. 84	I	1	2	1	2	1	2	2	2	(4)	—	(4)	—	(9)	—	2
S. 44	,,	1	1	1	1	1	1	2	3	(3)	—	(4)	—	(7)	—	1
S. 96	,,	1	1	1	1	1	2	1	2	3	4	(4)	—	(7)	—	1
S. 13	,,	1	2	2	3	2	4	4	5	(3)	—	(4)	—	—	—	3
G. 82	,,	2	3	2	3	2	3	2	4	(3)	—	—	—	—	—	3
OJ	,,	1	3	2	3	2	3	3	3	(3)	4	(3)	—	—	—	3
3444	,,	1	2	2	2	2	2	3	4	(4)	—	(5)	—	(5)	—	2
S. 14	,,	1	1	1	1	1	1	2	2	7	7	—	—	—	—	1
Peach 2	II	1	—	2	—	2	—	(1)	—	(1)	—	(1)	—	1	—	—
Venus 1	,,	1	—	2	—	2	—	2	—	2	—	2	—	2	—	—
S. 23	,,	1	—	2	—	2	—	1	—	2	—	2	—	(9)	—	—
Lottie 8	III	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1
M. 20	,,	1	—	3	—	4	—	(3)	—	2	—	2	—	1	—	—
Str. inulinaceus	,,	1	—	1	—	1	—	1	—	1	—	2	—	(3)	—	—
Str. bovis 1	,,	2	—	4	—	(4)	—	(4)	—	(4)	—	(4)	—	—	—	—
Str. bovis 2	,,	2	2	2	2	2	2	2	3	(4)	—	(4)	—	—	—	2
Str. thermophilus	,,	2	2	2	2	2	2	2	2	2	2	4	5	—	—	2

The first figure gives the number of days required to reduce the dye (brackets indicate only partial reduction attained). The second figure (and that in the last column) gives the number of days required to clot the milk. Temperature 37–38° C.

Note. The concentration of dye in the official method (3) has recently been found to be 1 in 550,000, not 1 in 300,000 as was generally thought (11).

Our solution was prepared from the purest obtainable dye supplied by G. T. Gurr.

(2) Other organisms

The fact that mastitis frequently results in high udder counts, and that these are due to cocci, suggests that these accompanying organisms may be responsible for the shorter reduction times obtained with some samples of mastitis milk. Wilson *et al.* (3) found that cocci were among the most powerful reducers of methylene blue and we have found that many of the cocci isolated from the milk of infected cows ferment lactose and partially reduce litmus, which has a much lower *r*H than methylene blue (five as compared with fourteen). It is possible, therefore, that the shorter reduction time associated with mastitis is related to the presence of these cocci. In market milk reduction is generally brought about by reducing types of cocci, coli-aerogenes bacteria and streptococci of the *lactis* type (3). In order to throw light on the problem the relation between count and reduction time was investigated.

The correlation between total count and methylene blue reduction time (single cow samples)

This correlation is shown in Table II.

It will be seen that, apart from the large number of samples negative to both tests, the correlation is very poor. It is concluded, therefore, that other factors play an important rôle in the reduction of methylene blue.

Table II

Methylene blue reduction time

Total count	++	+	(+)	0	Total
++	15	4	7	23	49
+	3	3	5	9	20
(+)	2	2	6	7	17
0	15	20	77	351	463
					549

For explanation of groupings see Table I (p. 75 of this *Journal*).

(3) Leucocytes and other cells

In milk taken from infected cows the leucocyte count is usually over 500,000 and frequently reaches many millions(4, 5, 6). Milk from mastitis-free animals usually has a leucocyte count of less than 50,000. Leucocytes can reduce methylene blue although the poising capacity is small, e.g. in the absence of oxygen leucocytes rapidly reduce methylene blue which, however, may be easily re-oxidized by the admission of a little air.

Thus it is sometimes found with milk from severely infected cows that on performing the methylene blue test, the dye is instantaneously reduced or almost reduced. The colour usually returns later, especially on inversion of the tube. The final true reduction time may be quite long, in fact, as long as that of normal milk. This phenomenon is invariably correlated with a high catalase figure and leucocyte count. Thus, while an excessive number of leucocytes (which may inhibit bacterial growth by phagocytosis(7)) may delay the *final true* reduction time caused by bacterial growth, a number greater than normal may assist in the reduction which is brought about later by the growth of the "secondary invaders" or cocci which frequently accompany the mastitis.

In order to test this hypothesis the relation between methylene blue reduction time and cell content was investigated. The catalase figure was used as a convenient numerical index of the cell content, as the catalase activity is due chiefly to leucocytes and body cells, together with blood (if present).

The correlation between methylene blue reduction time and the catalase figure

This is illustrated in Table III.

The correlation is quite high although definitely one-sided. Thus of thirty-six samples giving short reduction times, none was negative to the catalase test and thirty-two definitely positive.

Of ninety-six samples positive to the catalase test only thirty-two were positive to the methylene blue test. It may be concluded therefore that short reduction times are definitely linked with a high cell content but that milk

Table III

Catalase figures

Methylene blue reduction time	++	+	(+)	0	Total
++	20	3	1	0	24
+	8	1	3	0	12
(+)	13	9	9	42	73
0	22	20	42	144	228
					337

may have a high cell content without giving a short reduction time. Short reduction times may be brought about by the stimulation of actively reducing types of cocci by the increased number of cells in the milk. This factor may also be the reason why the methylene blue reduction test is unreliable for the bacteriological testing of milk from individual cows. Thus, it has been stated that "the milk of certain cows may have an abnormally high reducing activity even in the absence of any large numbers of bacteria" (3), p. 379).

(4) Chemical changes in the milk

Mastitis may result in alteration of the composition of the milk, in the severer cases to a considerable extent. This may affect the reduction time in two ways:

(1) By a reduction in the concentration of those substances which themselves may play a role, although a small one, in the reduction of the dye, e.g. hypoxanthine and xanthine oxidase (8).

(2) By a decrease in substances such as ascorbic acid, riboflavin, aneurin, etc. which are known to be important in the growth and metabolism of bacteria, especially the lactic acid bacteria (9) which are generally strong reducers.

It is evident that no general assumption as to the effect of mastitis can be made. What evidence is available, however, suggests that significant changes in the chemical composition of milk as the result of mastitis will lead to a lengthening of reduction time because of:

(1) The generally lowered nutritive content of the milk,

(2) Presence of increased amounts of bacteriostatic substances associated with globulin, and

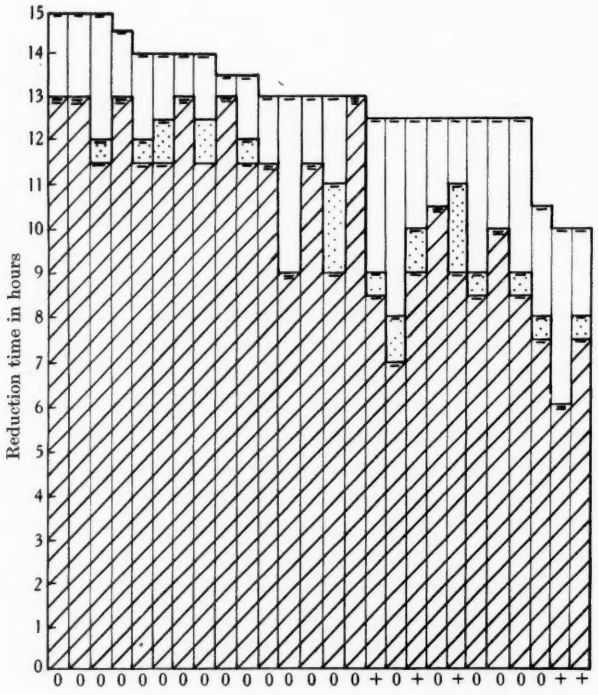
(3) Decreased amounts of substances known to act as bacterial growth factors.

We may now consider the usefulness of the methylene blue test for diagnosing mastitis.

Table IV

Methylene blue reduction time

"Diagnosis"	++	+	(+)	0	Total
++	16	13	19	52	100
+	4	3	11	38	56
(+)	2	3	11	51	67
0	5	7	50	190	252
					475



0 = Sample not infected;
+ = Sample from infected cows.

The height of the column represents the reduction time when tested immediately. The shaded portions represent the times after storage at 15·5 °C for 16 hr. The dotted portions represent the additional time required when a storage temperature of 4° C. was used.

Fig. 1. Effect of storage at 15·5° C. and at 4° C. on reduction times.

Diagnosis compared with the methylene blue reduction test

The test was made on mixed quarter samples about 3 hr. after milking, and readings taken up to 12–18 hr. The times bear no relation to those obtained in the official test as the conditions were quite different.

The results obtained are given in Table IV.

It may be seen that of 156 moderately and severely infected cows only thirty-six (23%) gave a short reduction time (<9 hr.). Of 252 samples free from infection, twelve gave a false positive reaction (5%). It is concluded therefore that while some cases of mastitis lower the reduction time, the test in its official forms is of no use for the detection of mastitis.

Effect of storage of samples at 4° *and* 15·5° C.

In an attempt to increase the efficiency of the methylene blue test for detecting mastitis, the samples in one series were divided into three parts. One was tested immediately as usual, a second stored at 4° C. overnight and a third at 15·5° C. overnight (about 16 hr.). The stored samples were then tested the next morning. The results are given in Fig. 1.

It will be seen that storage had no significant effect on the test. The reduction times were reduced roughly by one-quarter, but not much difference was found between the samples stored at 4° and those at 15·5° C. The difference was never greater than 2 hr. and only twice greater than 1 hr.

REFERENCES

(1) EDWARDS (1932). *J. comp. Path.* **45**, 43.
(2) DAVIS (1938). *J. Dairy Res.* **9**, 85.
(3) WILSON *et al.* (1935). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 206.
(4) DAHLBERG *et al.* (1936). *10th Ann. Rep. N.Y. Sta. Dairy Milk Insp.* p. 195.
(5) HUCKER (1933). *Amer. J. Pub. Hlth*, **23**, 237.
(6) CHERRINGTON *et al.* (1933). *J. Dairy Sci.* **16**, 59.
(7) WHITEHEAD & COX (1933). *Biochem. J.* **27**, 951.
(8) TWIGG (1937). *J. Soc. chem. Ind., Lond.*, **56**, 129 T.
(9) ORLA JENSEN (1936). *Zbl. Bakt.* II, **94**, 434.
(10) HOBBS (1939). *J. Dairy Res.* **10**, 35.

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213. STUDIES IN MASTITIS

V. MASTITIS IN RELATION TO CHEESE-MAKING

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(With 1 Figure)

Instances of the deleterious effect of "mastitis" on milk for cheese-making have been recorded over a considerable period. Until recently it was neither possible to define what was meant by mastitis nor to consider it in its chemical and bacteriological aspects. Many of the early reports must therefore be accepted with caution, a point emphasized in a review by Davis & Mattick (1).

The attitude of a cheese-maker towards mastitis must be rather different from that of a producer who supplies the liquid market. Milk may be suitable for consumption in the liquid state and yet be quite unsuitable for cheese-making. It follows, therefore, that the cheese-maker who wishes to eliminate faulty milk must employ different tests from those of the producer who is interested only in the prevention or eradication of mastitis. In other words the behaviour of the milk in the cheese vat cannot be predicted by a mere examination for the presence of *Str. agalactiae*.

From the cheese-making point of view, milk may be unsuitable for the following reasons:

(1) factors or conditions resulting in "slow starter";

(2) slow coagulation with rennet and formation of weak curd;

(3) contamination with common fault-producing organisms resulting in off-flavoured cheese;

(4) the presence of organisms which produce the more rare and late-developing faults such as "red spot" and "stinker";

(5) abnormal chemical composition which may affect structure and ripening generally; and

(6) abnormal enzyme content which may affect both chemical ripening and the development of flavour.

A search has therefore been made for a simple test which, besides being an efficient test for mastitis, will give a reasonably accurate indication of the suitability of the milk for cheese-making. Ultimately such a test was developed. It may be described as the *BCP-rennet test* (BCP = brom-cresol-purple).

THE EFFECT OF MASTITIS ON THE RENNETING POWER OF MILK

The rennet test by itself, although the best single test for the cheese-maker, is, as a test for mastitis, neither better nor worse than other indirect tests (Hadley (2)). A comparison of the test with the standard technique (diagnosis in Edwards's medium) was made and the results are given in Table I.

Table I

Slow clotting with rennet

"Diagnosis"	++	+	(+)	0	Total
++	7	3	4	12	26
+	0	3	1	4	8
(+)	0	3	2	23	28
0	2	6	16	72	96
					158

Thus of thirty-four moderately and badly infected cows thirteen gave a positive rennet test (38%) and of ninety-six uninfected animals eight were abnormal to rennet (8%). Our results thus confirm those of Hadley (2). As a matter of interest the relation between total count on milk agar and renneting power was investigated and the correlation found is shown in Table II.

Table II

Slow clotting with rennet

Total count	++	+	(+)	0	Total
++	8	0	1	2	11
+	1	0	0	1	2
(+)	2	2	0	3	7
0	7	22	37	149	215
					235

It will be seen that half the samples with very bad renneting times had high counts, whereas a negligible number of normally clotting samples were badly infected. It is unlikely, of course, that the high counts are directly responsible for the slow rennet test. The figures show, however, that high count milk may be bad for cheese-making for two reasons—the presence of taint-producing micro-organisms and poor clotting with rennet. The latter, leading to slow drainage and high moisture, thus favours the former.

The rennet test is one of the oldest tests known and is perhaps the best single test for abnormality in a milk as it is influenced by *p*H, calcium content

and casein-globulin ratio. The BCP-rennet test which will now be described is very simple, can be carried out with the same apparatus as the methylene blue reduction test and yields the following information:

(1) brom-cresol-purple reaction,
(2) clotting time with rennet,
(3) type of curd,
(4) appearance of curd after incubation (16 hr. at 37° C.),
(5) presence of *Str. agalactiae* (by microscopic examination after incubation),
(6) development of off-taints (by smell).

Description of the BCP-rennet test

Apparatus and material required

Rennet solution. Incorporate 0·2 ml.[1] of a fresh supply of a good commercial rennet in 100 ml. of 0·04% brom-cresol-purple solution. Brom-thymol-blue may be used if desired, but we have found brom-cresol-purple to be better for this purpose.

Tubes. British Standards Institution specification 152/16(3). A ring is etched at 10 ml. The exact size of these tubes is not important but it is advantageous to use the same tubes as those for the methylene blue test. The tubes are fitted with rubber bungs and both should be sterile.

Water bath at 37° C. It is an advantage to use a thermostatically controlled bath such as that laid down for the methylene blue test(3).

Method

The tube is filled to the 10 ml. mark with the sample and 1 ml. of the special rennet solution added and mixed by inversion of the tube after insertion of the rubber bung. From twenty to thirty tests should be done at a time.[2]

The colour of the brom-cresol-purple is recorded in the first column of the record sheet and then the tubes placed in the bath at 37° C. and the time

[1] Under our conditions this concentration yielded a clotting time of 20–30 min. for normal milk. The following factors influence the clotting time of milk:
(1) amount and strength of rennet,
(2) season of the year,
(3) age of milk on testing (loss of CO_2 and change in proteins and proteose fractions),
(4) temperature,
(5) *p*H,
(6) calcium content (both soluble and insoluble),
(7) casein, albumin and globulin content.
It will be obvious that a standard cannot be laid down and that twice or even three times the amount of rennet may be required to clot normal milk in 20–30 min. This point is not important however, as the abnormal samples are readily detected irrespective of the amount of rennet used.

[2] Less than twenty samples may fail to yield a good "spread" of fast, normally and slow clotting milks. More than thirty will entail a longer time in setting up and so introduce errors into the time measurements.

noted. The contents are examined for clotting after $\frac{1}{4}$, $\frac{1}{2}$, 1, 3 and 16 hr. by gently tilting the tube.[1] Care must be taken to avoid shaking the tube, especially if the milk has started to clot. For routine testing at a farm or factory it is sufficient to examine after 3 hr. After 16 hr. the tubes are examined for smell and a smear of the milk made if microscopic examination is to be carried out.

INTERPRETATION OF RESULTS

Assuming that the strength of rennet is adjusted to clot normal milk in 20–30 min. the results may be interpreted as follows:

(1) *Samples clotting in* 15 *min.* Very rapid clotting indicates either a form of mastitis resulting in acid milk or milk from a recently calved cow. Colostrum is quite acid to brom-cresol-purple (yellow-green) and milk from recently calved cows rather more acid than normally (greenish grey to brom-cresol-purple). If the clot does not break down in 16 hr. (i.e. does not undergo digestion with expression of whey) and has not developed any objectionable smell, the milk may be passed as fit for cheese-making. Colostrum (i.e. milk up to the fifth day after calving) should, of course, never be used for cheese-making, and it is assumed that it would not be tested by this method.

(2) *Samples clotting in* 16–30 *min.* Practically all samples of sound milk will fall in this group and give a pale slate-grey colour, tinged with blue, with brom-cresol-purple.

(3) *Samples clotting in* 31–60 *min.* These samples, if not from late lactation cows, may be regarded as suspect or slightly abnormal. They sometimes give a slightly alkaline reaction with brom-cresol-purple (a slightly purplish grey). Milk from late lactation cows gives a grey tinged with blue rather than purple (i.e. it contains less red).

Provided that no marked digestion or objectionable smell develops in 16 hr. the milk may be used for cheese-making.

(4) *Samples clotting in* 61–180 *min.* These milks are definitely abnormal and it is theoretically advisable to exclude them from the cheese-vat. However, the number of such samples commonly found makes it uneconomic to reject this milk. There is no evidence to show how far the proportion of such milk commonly occurring in bulk milk affects the cheese-making. These milks are usually alkaline to brom-cresol-purple (purple grey) and rarely give a good firm clot.

(5) *Samples failing to clot in* 180 *min.* Milks falling in this group should never be used for cheese-making. The slow renneting (or failure to clot, as these samples frequently fail to clot even on prolonged standing) is usually associated with a high globulin content, resulting in slow coagulation, weak curd, running of whey, etc. This type of milk may affect normal milk. Thus in one experiment a 20% addition of such milk to normal milk increased the

[1] These times are intended only as a guide. Prevailing conditions may require adjustment of the strength of the rennet solutions or of the times of observation.

clotting time by 50%. Such milk may not be markedly alkaline. The brom-cresol-purple test alone, therefore, is not adequate as a test for the cheese-maker.

Final examination

The samples may be left at 37° C. overnight and the curd examined next morning. Normal milks give a firm clot with a little expression of whey after 16 hr. Some samples (mostly the abnormal ones) undergo marked digestion with expression of whey. The factors controlling this digestion have not yet been fully investigated. Very bad samples become yellow or yellow-green with a flaky digested curd. Examination by smell and smears of the curd or milk for microscopic examination may be made if desired. We prefer Gram's stain for this purpose.

Summarized interpretation of tests

Any milk which is:

(*a*) strongly alkaline (i.e. purple) to brom-cresol-purple, or

(*b*) fails to clot in 3 hr. when normal milk clots in 20–30 min., or

(*c*) becomes unduly digested in 16 hr., or

(*d*) develops an objectionable odour in 16 hr.,

should not be used for cheese-making.

Chemical composition of milks abnormal to rennet

It was frequently noticed that milk might be normal or only slightly alkaline to brom-cresol-purple and yet clot very slowly or not at all with rennet. Estimation of globulin and calcium (kindly carried out by Dr E. C. V. Mattick) showed that failure to clot was related to high globulin and low casein and calcium. Typical data are given in Table III.

Table III. *The effect of mastitis on renneting power, globulin, calcium and lipase*

Cow	Quarters	Diagnosis	Rennet test	Brom-cresol-purple test	Globulin (% of total N)	Calcium (% in milk)	Lipase figure (4, 5)
441	(RF, LH, RH)	+ +	+ +	0	8·27	0·089	—
365	(LH and RH)	+ +	+ +	+	15·66	0·098	6·9
365	(LF and RF)	0	?	+	5·66	0·144	14·5
302	Three	0	0	?	4·84	0·148	26·6
302	One	+ +	?	?	5·96	0·139	18·7
365	(LF and RF)	0	0	—	4·54	0·128	32·8
365	(LH and RH)	+ +	?	—	17·74	0·089	9·3
289	(RH)	+ +	+ +	?	9·05	—	—
RR 27	LF	+	?	?	—	—	27·0
RR 27	RH	+	+	+	—	—	20·8
Fillpail	RF	0	?	?	—	—	36·8
	LH	+	?	0	—	—	29·2

Mastitis in relation to enzyme content of the milk

The subject of the enzymes in milk in relation to cheese-ripening has not received much attention, but it is probable that flavour, especially that associated with the higher volatile acids in non-mould-ripened cheese, is

influenced by the natural lipase content of the milk. E. C. V. Mattick has shown that mastitis definitely lowers the lipase content of milk (see Table III)(5).

Mastitis in relation to cheese "body" (viscosity and elasticity)

Experienced cheese-makers have often stated that mastitis results in poor texture and weak body (cf. also(1)) although experimental evidence has hitherto been lacking. Certain experimental cheese made at this Institute(6) were available for rheological investigation. It was found that when milk

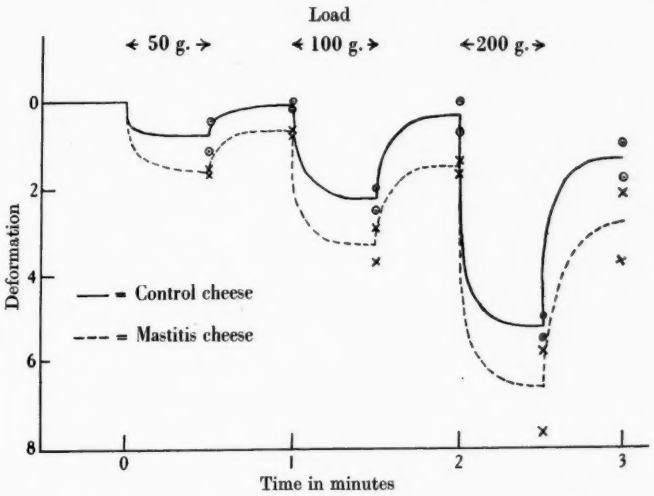

Fig. 1. Deformation and recovery curves of cheese made from normal and infected milk.

obtained from infected cows but not appreciably abnormal in composition was made into cheese, a product not significantly different in its rheological properties from cheese made from normal mastitis-free milk was obtained. Severer mastitis, resulting in definitely abnormal milk, yielded cheese of appreciably weaker "body" (lower viscosity but similar elasticity) than normal. This is illustrated in Fig. 1 which gives deformation curves obtained under identical conditions for the "mastitis" and control cheese. Analytical figures for the cheese obtained by Dr W. L. Davies are given in Table IV.

Table IV. *Analytical data of cheese quoted in Fig.* 1

	Control	Mastitis
	% of cheese	
Moisture	36·80	34·50
Salt	0·89	0·87
Ash	2·78	2·33
Calcium	0·735	0·647
Phosphorus	0·477	0·440
	% of total nitrogen	
Non-protein nitrogen	18·6	17·2

It would appear that low ash and calcium in cheese are related to weak body.

Mastitis in relation to "red spot" in cheese

During their investigation of "red spot" in Cheddar cheese, Davis & Mattick (7) observed the marked stimulating action of certain bacteria and of mastitis milk on pigment production by *L. rudensis*, the cause of "red spot". Further, the organism was repeatedly isolated from the fore milk of certain cows which were recognized by their owners to have "bad quarters". Because the importance of mastitis in relation to milk products was at the time not appreciated, the significance of these observations was not realized. In the light of the present investigation it would appear that removal of infected cows from the herd would probably have eradicated the trouble or at least reduced it considerably. Unfortunately no opportunity of testing this theory has occurred.

Mastitis and "slow starter"

Mastitis has been alleged, often without satisfactory evidence, to be a cause of slow starter (8, 9). Milks which were tested for mastitis by the method described were also frequently tested for starter growth in order to examine the correlation between the presence of mastitis (or other abnormalities) and the inability of the milk to permit normal growth of starter.

Technique

Ten ml. of the sample were transferred aseptically to a sterile plugged tube, and 1 ml. of a 10% suspension of a fresh milk culture of *Str. lactis* or *Str. cremoris* in saline or $\frac{1}{4}$-strength Ringer solution added and well mixed in. The tubes were then incubated at 30° C. (or at 22° C. overnight and transferred to 30° C. in the morning) and examined at suitable intervals for clotting. It is naturally impossible to lay down a standard time which normal milk will require to clot. Under our conditions about 20 hr. were required. The relationship between rate of starter growth and the presence of *Str. agalactiae* is illustrated in Table V.

Table V

Slowness of starter growth

"Diagnosis"	++	+	(+)	0	Total
++	6	14	3	10	33
+	0	5	3	8	16
(+)	2	8	2	23	35
0	4	15	24	83	126
					210

Thus, considering the effect of varying degrees of infection (as indicated by numbers of *Str. agalactiae*), it will be seen that twenty of thirty-three samples

from severely infected cows (61%) gave markedly slow growth with starter (++ and +). Similarly five of sixteen moderately infected samples (31%), ten of thirty-five slightly infected (29%), and nineteen of 126 samples from uninfected cows (15%) gave a slow rate of starter growth.

It was noticeable that the "spread" of times of clotting in these experiments was much less than that of the renneting times. Thus the arbitrarily defined + in the groupings of "slowness" varied from 110 to 150% of the time for clotting in normal milk, e.g. if 20 hr. were the limit for a 0 standard, the + standard would vary from 22 to 30 hr. Only three of the 210 samples investigated in Table III failed to give a clot in 200% of the time required for clotting in normal milk (40 hr.) and these samples were obviously abnormal (presence of blood or milk brownish in colour). It would seem therefore that although milk from infected cows undoubtedly causes slowness of starter growth in some instances, the dramatic hold-up experienced on some farms and in factories (where a delay of many hours occurs) cannot be ascribed to ordinary forms of mastitis. The instances quoted by some investigators where milk from one animal may hold up the development of acidity in the whole bulk would appear to be rare (9). Further, it has been shown that blood and serum do not appreciably affect the rate of growth of starter organisms in milk (10).

The relation between slow rennet action and slow starter growth

A high correlation was found to exist between slow rennet action and slow growth of starter. It will be evident therefore that in practice systematic use of the rennet test will detect those milks likely to cause starter trouble in all but rare cases (Table VI).

Table VI

Slowness of starter growth

Slow renneting	++	+	(+)	0	Total
++	5	15	0	0	20
+	1	6	5	12	24
(+)	3	14	5	23	45
0	6	26	34	111	177
					266

It will be observed that all samples which gave a very bad rennet test gave slow growth of starter, while only a few which were normal to rennet gave markedly slow starter growth.

Further, of these 266 samples only six gave a faster rennet time than normal (not differentiated in table) *and* slow growth of starter, and only one sample a slow rennet time *and* a faster growth of starter than normal (not differentiated in table).

There is little doubt, therefore, that the rennet test is a reliable method of assessing milk for starter growth.

Mastitis in relation to vitamin and bacterial growth factor content

Estimations of vitamin B_1 (aneurin), B_2 (lactoflavin) and C (ascorbic acid) in normal and mastitis milk have shown that mastitis significantly lowers the content of the milk in these substances (Table VII). The importance of at least some of these for the growth of starter organisms is recognized (13) so that the correlation between mastitis and slow growth of starter may be related to a low content in bacterial growth factors.

Table VII. *The vitamin contents of normal and mastitis-infected milk*

Vitamin	Normal	Infected	Reference
Aneurin	7·53 I.U./100 ml.	4·31 I.U./100 ml.	(11)
	7·52 ,,		
	7·21 ,,		
Lactoflavin	151 μg./100 ml.	50 μg./100 ml.	(11)
	128 ,,	74 ,,	
Ascorbic acid:			
Reduced	2·19 mg./100 ml.	1·44 mg./100 ml.	(12)
Total	2·48 ,,	1·87 ,,	
Reduced	2·43 ,,	1·05 ,,	(11)
		1·35 ,,	
		1·35 ,,	
Total	2·51 ,,	1·11 ,,	
		2·00 ,,	
		1·72 ,,	

All infected samples were macroscopically normal.

As a point of interest and in view of the general employment of the methylene blue reduction test, the correlation between the reduction time and the rate of starter growth was investigated. The data are given in Table VIII.

Table VIII

Slowness of starter growth

Methylene blue reduction time	++	+	(+)	0	Total
++	4	9	0	0	13
+	1	5	6	10	22
(+)	2	6	6	17	31
0	5	39	34	120	198
					264

It will be seen that a very similar picture to that given by the rennet and starter growth data is obtained. All samples reducing methylene blue in less than 3 hr. gave slow starter growth.

The correlation between total count and growth rate of starter

The data for this correlation are given in Table IX. It will be seen that high counts are definitely associated with slow growth of starter. In view of the known stimulating action of most micro-organisms on starter streptococci

it may be assumed that this apparent relation is really a consequence of the correlation between total count and abnormal chemical composition.

Table IX

Slowness of starter growth

Total count	++	+	(+)	0	Total
++	5	7	1	1	14
+	0	1	0	2	3
(+)	1	2	1	1	5
0	6	49	44	142	241
					263

Observations on actual cheesemaking experiments are discussed elsewhere (14).

REFERENCES

(1) DAVIS & MATTICK (1936). *Agric. Progr.* **13**, 126.
(2) HADLEY (1936). *J. Dairy Sci.* **19**, 165.
(3) MEMO 139/FOODS (1937). H.M. Stationery Office.
(4) MATTICK, E. C. V. & KAY (1938). *J. Dairy Res.* **9**, 58.
(5) MATTICK, E. C. V. (1938). Unpublished results.
(6) MATTICK, A. T. R., DAVIES & DEARDEN (1937). *Proc. World's Dairy Congr.* 1937, 1.
(7) DAVIS & MATTICK (1931). *J. Dairy Res.* **2**, 190.
(8) DAVIS (1935). *Agric. Progr.* **12**, 138.
(9) LEITCH (1937). *Agric. Progr.* **14**, 40.
(10) DAVIS (1939). *J. Dairy Res.* (in the Press).
(11) HOUSTON & KON (1938). N.I.R.D. unpublished results.
(12) KON & WATSON (1937). *Biochem. J.* **31**, 223.
(13) ORLA JENSEN *et al.* (1936). *Zbl. Bakt.* II, **94**, 452.
(14) DAVIS (1939). *Agric. Progr.* **16** (in the Press).

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214. STUDIES IN MASTITIS

VI. GENERAL OBSERVATIONS, SUMMARY AND CONCLUSIONS

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General observations

During the course of this investigation data were accumulated which afforded information upon some points of general interest.

The correlation of incidence of mastitis with age of the cow

It is generally recognized that older cows (e.g. those having had three or more calves) are more prone to mastitis than younger animals. Indeed, there is an impression amongst some practical workers that first calf heifers never, or very rarely, suffer from udder disease. Our findings in this connexion are given in Table I.

It will be seen that the incidence of mastitis (++ and +) in first calf heifers on the farms studied is 22%, in cows with two calves 37% and in those with three or more calves 44%. There is thus a marked rise in incidence with the second calf and an appreciable incidence in first calf heifers, contrary to general belief. If the (+) results are included (the abnormality of these samples is only rarely detected by the simple tests) the figures become 36, 50 and 57%.

Table I. *The relationship between number of calves and the incidence of mastitis*

"Diagnosis"	Number of calves: 1	2	3 or more	Total
++	14 (9%)	45 (24%)	52 (36%)	111
+	20 (13%)	25 (13%)	11 (8%)	56
(+)	22 (14%)	25 (13%)	19 (13%)	66
0	96 (64%)	93 (50%)	62 (43%)	251
Total	152	188	144	484
++ and +	22%	37%	44%	

THE CORRELATION BETWEEN AGE OF COW AND TOTAL BACTERIAL COUNT OF THE MILK

There is a belief amongst dairy bacteriologists that older cows have higher udder counts than younger cows. The relation between age and udder count is illustrated by the data given in Table II.

Table II. *The relationship between age and udder count for infected and non-infected animals*

Diagnosis	Udder count	Number of calves		
		1	2	3 or more
+ +	+ +	5	15	19
	+	0	6	6
	(+)	1	4	4
	0	8	20	23
+	+ +	3	3	3
	+	0	1	0
	(+)	0	1	1
	0	17	20	7
(+)	+ +	0	1	0
	+	0	0	0
	(+)	1	3	0
	0	21	21	19
0	+ +	0	6	0
	+	0	4	1
	(+)	2	1	2
	0	94	88	59

These data show beyond doubt that only when a cow is severely infected with mastitis is there a likelihood of an increased count due to age as distinct from an increase due to a higher incidence of mastitis. The higher counts found in older cows are due entirely to mastitis. Provided that mastitis is absent, therefore, an old herd should be capable of giving milk containing as few bacteria as that from a young herd.

THE SIGNIFICANCE OF STAGE OF LACTATION IN RELATION TO INCIDENCE OF MASTITIS AND TOTAL COUNT ON MILK AGAR

In Table III are given data showing the relation of stage of lactation (i.e. whether in full milk or in late lactation according to our definition given earlier) to mastitis and total count.

Table III. *The relationship between stage of lactation and udder count for infected and non-infected cows*

Total count	Cows in full milk	Cows in late lactation
	Mastitis-infected cows	
+ +	40 (32%)	8 (21%)
+ and (+)	19 (15%)	4 (11%)
0	65 (53%)	26 (68%)
	Mastitis-free cows	
+ +	0	0
+ and (+)	5 (3%)	4 (8%)
0	195 (97%)	44 (92%)

The data are perhaps too scanty to permit of definite conclusions, but there is a suggestion that of mastitis-free cows, those in late lactation have a slightly higher count, and of infected cows, that those in late lactation have a somewhat lower count.

Summary of papers I–VI

I. Methods of taking and examining samples of milk for *Str. agalactiae* are described.

Where delay in examination is unavoidable Edwards's (1) medium is recommended. This selective medium, however, does not permit the growth of some types of organisms which may cause a subclinical mastitis. It is therefore recommended that, where possible, samples be examined immediately after taking and a non-selective medium used. In this way a measure of the udder count is obtained which is of great value. Where samples can be examined within a few hours plain blood agar is preferred. If blood is unobtainable ordinary milk agar may be used. Non-blood media have certain advantages over blood media, which compensate for the loss of ability to detect haemolysis when it occurs.

A simple confirmatory test for *Str. agalactiae* is described. Confirmation of type is regarded as essential in all but the obvious cases.

II. The results of a number of simple tests for mastitis have been compared with those from examination in blood agar. These all have roughly the same order of efficiency in that they detect about half the positive cases and give 10–30% false positives. It is suggested, therefore, that with two exceptions (the milk agar count and the rennet test, which may be used for special purposes) they are so unreliable as not to be worth doing in any laboratory examination.

The brom-cresol-purple paper, strip cup and induration tests, which are very simple and can be carried out by the milker, are worth doing in order to get a general idea of the incidence of mastitis in the herd. Their limitations should be realized, however, for no simple test, or combination of simple tests, is really satisfactory, especially if the owner wishes to secure eradication of the disease from the herd. Our results thus confirm those of other workers.

III. Mastitis has a marked effect on the "udder count" as given by total colony count on milk agar. No samples from mastitis-free cows were found to have a count of over 2000 per ml. and very few over 500. About 50% of infected cows yielded milk with a count of over 1000.

The milk agar count is perhaps the best of the indirect tests as although it detects no more than other tests (about 50%) it gives very few false positives. A count of under 100 per ml. may be taken to indicate freedom from infection and a count of over 1000 to indicate mastitis.

IV. The methylene blue reduction test is not capable of detecting mastitis. Reduction time appears to be more closely related to the cell content of infected milks than to the total count. Storage of the samples for 16 hr., either at 4 or 15·5° C., did not materially improve the efficiency of the test.

V. The possible causes of the unsuitability of mastitis milk for cheese-making are classified and discussed. The brom-cresol-purple-rennet test is described and recommended as a general test for mastitis and suitability of the milk for cheese-making. The effect of mastitis on the chemical composition and enzyme content of the milk is discussed. Mastitis adversely affects the "body" of cheese and is an important factor in the fault known as "red spot". Many samples of mastitis milk give slow growth of starter, and these are usually detected by the rennet test. Deficiency in bacterial growth factors may be one factor in causing slow starter. This phenomenon is also correlated with rapid reduction of methylene blue and high total count, but the real factor responsible is probably abnormal chemical composition.

VI. The incidence of mastitis increases steadily with age of the animal. Older cows do not give milk of higher "udder count" than younger cows if infection is absent. The increased count is entirely due to the increased incidence of mastitis. There appears to be a somewhat lower udder count in late lactation in infected cows, but a slightly higher one if mastitis is absent.

Finally we may emphasize the fact that for eradication purposes there are only two methods of diagnosis worth using—simple tests on the farm and carefully controlled bacteriological examination in the laboratory.

ACKNOWLEDGEMENTS

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REFERENCE

(1) EDWARDS (1933). *J. comp. Path.* **46**, 211.

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215. THE VALUE OF INDIRECT METHODS FOR THE DETECTION OF MASTITIS

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The indirect methods for the diagnosis of mastitis are based on the detection of the changes which take place in the composition of the milk as a result of the infection. Such methods are liable to error on account of the natural variation in the composition of milk from uninfected quarters. This variation makes it difficult to fix, for the interpretation of the tests, standards which will prevent the tests from failing to detect a proportion of the infected quarters and, at the same time, from diagnosing some uninfected quarters as infected. The smaller the variation with which any particular indirect test is concerned, the more satisfactory that test will be.

In many cases of subclinical mastitis the changes in the composition of the milk are slight and will not be detected by an examination of the mixed milk from all four quarters of the udder if, as frequently happens, only one or two are infected. Hence negative results with mixed milk cannot be taken as definite and the indirect tests give their best results when applied to the milk from separate quarters.

This paper records briefly the results of the application of tests for the presence of clots, the reaction to brom-cresol-purple and brom-thymol-blue, the amount and character of the centrifuge deposit, the catalase content, the chloride content, the casein number and the solids-not-fat content to a series of milk samples from individual quarters. Cows in advanced lactation were not included as their milk shows most of the changes of mastitis milk, and makes cultural examination essential. The indirect tests were therefore investigated under the best possible conditions for their success. As the quarters were under strict bacteriological control, the data obtained show the relative value of the above indirect tests for the diagnosis of subclinical mastitis.

Experimental

The samples of milk were obtained from cows of the Shorthorn, Friesian, Ayrshire, and Guernsey breeds. The cows were in full milk and belonged to herds almost free from clinical mastitis.

Sampling and clinical examination

The first streams of milk from each washed and dried quarter were drawn into a black dish and inspected for the presence of clots. Then, after testing the reaction of the milk, about 20 ml. were drawn into a sterile tube for the

bacteriological and centrifuge deposit tests, followed by 2–4 oz. into a bottle for the chemical tests. The examination of most of the samples was commenced within a few hours; the remainder were stored at a low temperature.

After the milking was completed the udder was palpated for fibrosis.

Bacteriological examination

The centrifuge deposit from 10 ml. of milk was plated on the selective crystal violet aesculin blood agar medium of Edwards(1). The plates were examined for the presence of colonies of *Str. agalactiae* against a black background as suggested by Cunningham(2). Doubtful colonies were picked off to yeast dextrose litmus milk, incubated, inspected, and finally tested by catalase reaction and Gram staining.

Reaction to indicators

Brom-thymol-blue papers were prepared from filter paper dipped in a 0·065% solution of the indicator in 68% ethyl alcohol, dried, and cut into strips. Brom-cresol-purple papers were prepared similarly in an 0·080% aqueous solution. Two drops of fore milk were drawn on to the papers and the colours compared, after $\frac{1}{2}$–1 min., with that produced by an uninfected quarter in full milk, taken as a standard. The standard for brom-thymol-blue was a faint yellowish green colour, and greenish yellow and green colours were taken as positive. The standard for brom-cresol-purple was slate grey, and any tendency to purple was taken as positive. The tests were reported as doubtful when it was found impossible to decide definitely whether or not the colour was a little abnormal.

Centrifuge deposit

Ten ml. of the fore milk were centrifuged for 10–12 min. at 2500 rev./min. in a modified Trommsdorff tube, as described by Minett *et al.*(3), and the amount, colour and degree of separation of the deposit observed—a yellow colour indicates the presence of pus, and a red colour that of blood corpuscles —and interpreted thus: *positive* (1) if 0·01 ml. or more, or (2) if yellow; *negative* if not more than 0·005 ml., and of normal appearance, i.e. not slightly yellow, red, or well separated from the supernatant fluid; *doubtful* (1) if between 0·005 and 0·01 ml. and of normal appearance, or (2) if less than 0·01 and slightly yellow, or red, or well separated.

This scheme was the best that could be derived from the data obtained, in that it gave the minimum of doubtful reactions.

Catalase test

Fifteen ml. of milk plus 5 ml. of 1% hydrogen peroxide solution were incubated in an Einhorn saccharometer tube for 2 hr. at 37° C. The tubes were allowed to cool, and samples yielding more than 0·5 ml. of gas were considered positive. This is the limit usually adopted and there appeared to be no advantage in modifying it.

Chloride content

The chlorides were determined by the wet digestion method of Davies (4), and a content exceeding 115 mg./100 ml. was taken as positive, as this was found to be the most satisfactory figure. Limits of 115 and 120 mg. have been recommended previously (5,6,7), and Rosell's (8) limit of 140 mg. for "direct titratable chloride value" corresponds approximately with 112 mg. by accurate methods of chloride determination (9).

Casein number

The determination and application of this number $\left(\frac{\%\text{ of casein } N}{\%\text{ of total } N} \times 100\right)$ have been fully described and explained in a previous paper (10). A figure above 78·0 was taken as negative.

Solids-not-fat content

As mastitis has been shown to account for a high proportion of milk samples low in solids-not-fat content (11), this content may be considered as another possible means for the indirect diagnosis of mastitis. The total solids content of the samples was determined by evaporation and the fat content by the Gerber method. The solids-not-fat content was calculated as percentage of the fat-free milk (to avoid differences which might be due solely to the presence of varying amounts of fat) and a figure below 8·80 was taken to indicate a positive sample.

Results

The results of the indirect tests are summarized in Table I. It will be seen that the number of quarters examined by the different tests varied as the tests were in turn discontinued when sufficient evidence on any one had accumulated. In all 265 different quarters were sampled, of which 130 were found to be infected. Only 9, i.e. 7%, of these infected quarters showed clinical signs of mastitis.

The clot and brom-thymol-blue tests did not diagnose any of the uninfected quarters as infected, i.e. they gave no false positives, but they detected only 12 and 14% respectively of the infected quarters. The brom-cresol-purple test detected 61% of the infected quarters, and gave 16% of false positives. With the brom-thymol-blue it was found difficult to observe a colour change in the lower range of *p*H variation, and this accounts for the large proportion of quarters reported as doubtful, 51% of the infected, and 14% of the uninfected quarters. There is much difference of opinion among previous investigators with regard to the relative merits of brom-thymol-blue and brom-cresol-purple, in both paper and solution form, for the detection of mastitits. We found the papers to be better than the solutions, as they are less affected by the colour of the milk and the presence of fat; and we definitely prefer brom-cresol-purple as its colour change was easier to detect and it gave

Table I. *The accuracy of the clot, indicator, deposit, catalase, chloride, casein number, and solids-not-fat tests*

Result of indirect test	Bacteriologically positive quarters*		Bacteriologically negative quarters*	
	No.	% of total	No.	% of total
Clot test				
Positive	15	12	0	0
Negative	115	88	135	100
Brom-cresol-purple test				
Positive	58	61	19	16
Doubtful	26	27	26	22
Negative	11	12	74	62
Brom-thymol-blue test				
Positive	13	14	0	0
Doubtful	49	51	17	14
Negative	33	35	102	86
Centrifuge deposit				
Positive	38	55	2	3
Doubtful	16	23	18	25
Negative	15	22	51	72
Catalase content				
Positive	30	49	21	34
Negative	31	51	40	66
Chloride content				
Positive	53	77	0	0
Negative	16	23	38	100
Casein number				
Positive	116	91	9	8
Negative	12	9	106	92
Solids-not-fat content				
Positive	76	63	10	9
Negative	45	37	104	91

* Each quarter is included once only in each test.

better results. Theoretically, brom-thymol-blue, covering a *p*H range of 6·0–7·6, should be more suitable than brom-cresol-purple, covering a *p*H range of 5·2–6·8, for the detection of a change in the *p*H of milk.

The centrifuge deposit test, when interpreted as described above, detected 55 % of the infected quarters, and gave only 3 % of false positives. The catalase test detected only 49 % of the infected quarters, and gave 34 % of false positives. The chloride test detected 77 % of the infected quarters and gave no false positives. This absence of false positives was no doubt assisted by the avoidance of cows in advanced lactation. The results of previous investigations of the above tests have been extensively reviewed recently by Munch-Petersen (12).

The casein number detected the highest proportion of the infected quarters (91 %), and gave few false positives (8 %). These results have been discussed and the discrepancies considered in an earlier paper (10). The solids-not-fat test detected 63 % of the infected quarters, and gave 9 % of false positives.

Conclusion

The indirect tests for mastitis must be judged from both the proportion of infected quarters correctly detected and the proportion of uninfected quarters wrongly detected. Any attempt to assess the extent of the agreement between the indirect tests and the direct bacteriological findings, or to place the tests in the order of their value for diagnosis, is complicated by the two conflicting sets of figures which have to be dealt with.

For each of the indirect tests the "mean square contingency" has been calculated, as providing the best available measure of agreement between it and the cultural method, according to the formula:

$$\text{Mean square of contingency} = \frac{(ad-bc)^2}{(a+b)\,(c+d)\,(a+c)\,(b+d)},$$

where a = number of bacteriologically positive quarters found positive by indirect test,

b = number of bacteriologically negative quarters found positive by indirect test,

c = number of bacteriologically positive quarters found negative by indirect test,

d = number of bacteriologically negative quarters found negative by indirect test.

The result is given in Table II, where the figure 0 would indicate that there is no agreement, and the figure 1 perfect agreement.

Table II. *The agreement between the indirect tests and the bacteriological findings*

Indirect test	Mean square contingency
Casein number	0·698
Chloride content	0·541
Centrifuge deposit	0·335
Solids-not-fat content	0·314
Brom-cresol-purple reaction	0·218
Brom-thymol-blue reaction	0·085
Presence of clots	0·061
Catalase content	0·022

Summary

The following indirect tests for the detection of subclinical mastitis were applied, under bacteriological control, to a series of milk samples from the individual quarters of cows in full milk: presence of clots, reaction to brom-cresol-purple, reaction to brom-thymol-blue, amount and character of centrifuge deposit, catalase content, chloride content, casein number and solids-not-fat content.

These tests detected 12, 61, 14, 55, 49, 77, 91, and 63 % respectively of the infected quarters, and diagnosed 0, 16, 0, 3, 34, 0, 8, and 9 % of the uninfected quarters as infected.

For each indirect test the mean square contingency was calculated as a measure of agreement between it and the direct bacteriological method. The tests were placed in the descending order of their value for diagnosis thus: casein number, chloride content, centrifuge deposit, solids-not-fat content, brom-cresol-purple reaction, brom-thymol-blue reaction, presence of clots, catalase content.

REFERENCES

(1) EDWARDS (1933). *J. comp. Path.* **46**, 211.
(2) CUNNINGHAM (1936). "The occurrence and detection of streptococcal mastitis." *Proc. Soc. agric. Bact.*
(3) MINETT, STABLEFORTH & EDWARDS (1930). *J. comp. Path.* **43**, 163.
(4) DAVIES (1932). *Analyst*, **37**, 71.
(5) KRENN (1930). *Fortschr. Landw.* **5**, 413.
(6) SEELEMANN (1932). *Die streptokoken Infektionen des Euters.* Hanover: M. and H. Schaper.
(7) ZIEGER (1932). *Dtsch. tierärztl. Wschr.* **40**, 17.
(8) ROSELL & MILLER (1933). *J. Amer. vet. Med. Ass.* **82**, 587.
(9) BLOOD & ROWLANDS (1936). *J. Dairy Res.* **7**, 47.
(10) ROWLAND & ZEIN-EL-DINE (1938). *J. Dairy Res.* **9**, 174.
(11) —— —— (1938). *J. Dairy. Res.* **9**, 182.
(12) MUNCH-PETERSEN (1938). *Bovine Mastitis.* Imp. Bureau of Animal Health, Review Series, no. 1.

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216. THE EFFECT OF CHURNING ON THE VITAMIN A AND CAROTENE CONTENT OF MILK FAT

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During the normal churning of milk the fat is exposed to considerable aeration and it is quite conceivable that in the process there may be some loss of vitamin A and carotene by oxidation.

In the course of a recent investigation (Gillam *et al.*(1)) we had occasion to churn butters from a series of raw milks in the usual way and also to extract the fat from the same milks by means of a solvent. The fats so obtained afforded a good opportunity of studying the behaviour of vitamin A and carotene in the course of churning. In addition to a series of eleven samples produced by each method there were available another double sample (no. 12, Table I) churned and extracted from raw liquid milk from a different source and duplicate samples obtained from reconstituted roller-dried (no. 13, Table I) and spray-dried (no. 14, Table I) full-cream milk.

The fat extraction was carried out as follows: To 1 l. of milk in a large separating funnel were added in succession 700 ml. of anaesthetic ether, 50 ml. of alcohol, 25 ml. of a KOH solution of sp. gr. 1·26–1·27 and a further 150 ml. of alcohol. After each addition the mixture was well shaken. Rapid separation of the ethereal and watery layers occurred after the last addition of alcohol. The aqueous layer was drawn off and further extracted with 400, 200 and 200 ml. lots of ether respectively. The combined ether extracts were washed three times, each time with 250 ml. of water and dried over anhydrous sodium sulphate. The ether was then removed, the last traces being drawn off *in vacuo*. The extraction was almost quantitative, the yields of fat being only very slightly below the quantities expected from a parallel estimation of fat in the milk by the Gerber method.

The churnings were carried out on a small scale in our experimental dairy under conditions comparable with those obtaining in normal manufacture, unripened, unpasteurized, unsalted cream being used. The cream from about 1 gal. of milk was used for each churning, except with sample 12 (3 gal.) and samples 13 and 14 ($1\frac{1}{2}$ gal. each). The average time of churning was 20–30 min.

Table I. *The vitamin A and carotene content of samples of butter-fat churned from milk in the customary way and of samples extracted by means of ether*

	Vitamin A					
	Spectrophotometrically			Lovibond tintometer		
	mg. vit. A/100 g. fat*		Difference extracted-churned	Moore blue units/g. fat		Difference extracted-churned
Sample no.	Extracted	Churned		Extracted	Churned	
1	1·71	1·59	+0·12	57·0	62·0	−5·0
2	—	1·18	—	60·0	64·0	−4·0
3	1·52	1·45	+0·07	64·0	65·0	−1·0
4	1·34	1·36	−0·02	62·0	64·0	−2·0
5	1·33	—	—	62·0	62·0	0
6	1·45	1·24	+0·21	62·0	62·0	0
7	1·29	1·25	+0·04	60·0	60·0	0
8	1·37	1·42	−0·05	62·0	62·0	0
9	1·25	1·35	−0·10	62·0	62·0	0
10	1·30	1·14	+0·16	58·0	58·0	0
11	1·35	1·00	+0·35	51·0	51·0	0
12	1·20	1·28	−0·08	57·1	58·6	−1·5
13	1·28	1·18	+0·10	59·3	60·0	−0·7
14	1·28	1·19	+0·09	60·0	60·0	0
Average	1·36	1·29		59·7	60·7	
Difference: extracted-churned	+0·07			−1·0		
S.E.M.	±0·037			±0·43		
P‡	1:12 Not significant			1:29 Apparently significant		

	Carotene					
	Spectrophotometrically			Lovibond tintometer		
	mg. carotene/100 g. fat†		Difference extracted-churned	Moore yellow units/g. fat		Difference extracted-churned
Sample no.	Extracted	Churned		Extracted	Churned	
1	0·84	0·77	+0·07	20·2	20·5	−0·3
2	0·89	0·66	+0·23	20·8	19·1	+1·7
3	0·83	0·75	+0·08	22·0	20·5	+1·5
4	0·86	0·70	+0·16	21·8	20·5	+1·3
5	0·86	0·67	+0·19	21·5	21·7	−0·3
6	0·81	0·67	+0·14	21·5	21·5	0
7	0·79	0·72	+0·07	20·4	19·7	+0·7
8	0·77	0·71	+0·06	21·5	20·6	+0·9
9	0·74	0·78	−0·04	19·5	20·4	−0·9
10	0·78	0·58	+0·20	20·2	18·9	+1·3
11	0·58	0·47	+0·11	12·9	13·3	−0·4
12	0·70	0·60	+0·10	19·6	18·7	+0·9
13	0·72	0·59	+0·13	19·8	17·8	+2·0
14	0·71	0·62	+0·09	20·4	18·7	+1·7
Average	0·78	0·66		20·1	19·4	
Difference: extracted-churned	+0·12			+0·7		
S.E.M.	±0·019			±0·25		
P‡	About 1:15,000 Significant			1:71 Significant		

* Based on the assumption that vitamin A itself has an intensity of absorption at 328 mμ $E^{1\%}_{1cm.}$ = 1600 (in alcohol).

† These figures are calculated on the basis of $E^{1\%}_{1cm.}$ = 2200 for pure carotene in $CHCl_3$ at 463 mμ.

‡ P = probability that a mean difference at least as great as the observed difference would have arisen by random sampling from a homogeneous population.

The fats were examined for vitamin A and for carotene by Lovibond tintometer tests (K.M.H. and S.K.K.) and by spectrophotometric methods (A.E.G.) using the technique previously described (Gillam *et al.*(2, 1)).

The results and their statistical examination are given in Table I. Before discussing these results it would be well to make it quite clear that the effect of churning can only be compared with the effect of extraction by some other method. We are of course not aware of the changes in the vitamin A and carotene content of the milk which may have occurred during the extraction of the fat with ether and we can only assume that the effect of such extraction, involving very little exposure to oxidation, was negligible.

The vitamin A results are at first sight somewhat conflicting. The spectrophotometric method suggests a small loss (about 5%) as a result of churning, while the Lovibond tintometer technique indicates an even smaller difference (under 2%) in the opposite direction. Of these two figures the former is statistically non-significant ($P = 1:12$) according to the usual *t*-tests (Fisher, (3)), while the latter at first appears to be significant ($P = 1:29$). The scatter of the tintometer differences (col. 7 of Table I) is, however, somewhat anomalous and the usual statistical treatment is therefore not properly applicable. It is, indeed, doubtful whether the tintometer test for vitamin A is accurate to more than 6% for a single difference (corresponding to 1·6% for the mean of 14 differences). We therefore conclude that the differences observed are not significant by either method. As the differences are so small and as the two methods give indications in opposite directions, it is probably safe to say that the effect of churning on vitamin A content is negligible, not exceeding about 4%.

The position is much clearer with regard to carotene. Here both methods show a loss on churning. In both cases this is statistically significant, with heavy odds in the spectrophotometric measurements, and the effect itself is much greater than that indicated by either method for the vitamin A, i.e. 15% by one method and 3·5% by the other. It is, however, worth remarking that these two estimates (15 and 3·5%) are themselves significantly different ($P = 1:1250$). Since the spectrophotometric method measures the absorption of light of definite wave-length by the unsaponifiable fraction while the tintometer method measures the integrated transmission in the presence of the saponifiable matter, it is not altogether surprising that the numerical estimates differ. Nevertheless, both methods indicate loss, and although no quantitative estimate is possible, it appears that the qualitative conclusion can be accepted with confidence that churned butter fat actually contains less carotene than an equivalent quantity of extracted fat. On the assumption that there is no loss of either carotene or vitamin A in the course of ether extraction the figures obtained on the churned fat show that in butter carotene is more labile than vitamin A.

SUMMARY

The vitamin A and carotene content of 14 samples of butter fat churned from milk in the usual way has been compared in spectrophotometric and Lovibond tintometer tests with figures similarly obtained for the ether-extracted fat from the same milks. On the assumption that there is no loss of either carotene or vitamin A in the course of the ether extraction, it has been found that some carotene is lost in churning but that vitamin A is not appreciably affected.

We are greatly indebted to Miss D. V. Dearden for the churning of the butters.

REFERENCES

(1) GILLAM, HENRY, KON & WHITE (1938). *J. Dairy Res.* **9**, 17.
(2) GILLAM, HENRY & KON (1937). *Milk and Nutrition*, pt I, p. 45. Reading: Nat. Inst. Res. Dairying.
(3) FISHER (1934). *Statistical Methods for Research Workers*, 5th ed. Edinburgh: Oliver and Boyd.

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217. THE WHITE PARTICLES IN MATURE CHEDDAR CHEESE

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The appearance of white particles in mature Cheddar cheese has frequently been noted. Van Slyke & Price (1) described them as "a calcium soap, formed by the combination of calcium with some fatty acid derived from the fat by the action of some micro-organisms acting only at low temperatures". Quite recently Tuckey *et al.* (2) have shown by X-ray analysis that the white specks have the same crystal spacings as calcium lactate. This latter conclusion is confirmed by results which had been obtained at this Institute by examination of a quantity of the deposits picked out from a mature cheese. It was found that the specks were larger and more numerous in the fissures occurring in the body of the cheese. In the course of a day three workers were able to accumulate 0·9 g.

Analysis by micro or semi-micro methods gave the following results:

	%
Moisture	28·5
Protein	11·3
Fat	17·2
Lactic acid	35·1
Ash	14·1
Calcium oxide	11·9
Calcium oxide in ash	83·1

Calcium was thus the main constituent of the ash. The proportion of lactic acid present was only slightly smaller than that required to give calcium lactate with all the calcium present. The percentage composition of the material isolated may therefore be cited as:

	%
Moisture	28·5
Protein	11·3
Fat	17·2
Calcium lactate	42·5
	99·5

If the calcium lactate be taken as containing the usual 5 molecules of water of crystallization, the composition becomes:

	%
$Ca(C_3H_5O_3)_2 . 5H_2O$	60·0
Moisture	11·0
Protein	11·3
Fat	17·2

The residue other than calcium lactate was thus obviously cheese adhering to the specks as picked out from the matrix.

Summary

The white specks appearing in mature Cheddar cheese have been shown by analysis to be calcium lactate.

REFERENCES

(1) Van Slyke, L. L. & Price, W. V. (1927). *Cheese*, p. 275. New York: Orange Judd Publishing Co.

(2) Tuckey, S. L., Ruehe, H. A. & Clark, G. L. (1938). *J. Dairy Sci.*, **21**, 161.

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218. STARTER CULTURES FOR CHEESE MANUFACTURE

MAINTENANCE OF ACID-PRODUCING ACTIVITY IN CULTURES OF LACTIC STREPTOCOCCI

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Attempts to control the activity of cheese-starter cultures have been described in previous papers from this Institute. It has been shown that the main function of the starter culture is the production of lactic acid in the cheese vat at a regular rate from day to day. If full control over starter cultures could be realized in practice it would soon be possible to specify a procedure best suited to the manufacture of Cheddar cheese from pasteurized milk of a known chemical composition; even before such specifications were worked out the cheese-maker would be able, with a starter of constant activity, to adjust his making procedure by a process of trial and error. Hitherto, in the process of manufacture, cheese-makers have only been able to carry out the successive operations in the same time and at the same acidities from day to day for limited periods which were invariably terminated by a more or less sudden alteration in the activity of the starter. This alteration in vitality occurred in spite of all attempts to maintain constant conditions of culture. Even when cultures of single strains of streptococci were used (Whitehead & Cox(1)) in place of the mixed streptococcal cultures of commerce, the variability still persisted; indeed, with single-strain cultures complete failures were more frequent than ever (Whitehead & Cox(2)). It was finally shown that the trouble in the case of single-strain cultures was due to the development of bacteriophage within the culture (Whitehead & Cox(3, 4)). Nelson & Hammer(5) and Mazé(6) have since reported the isolation of phage responsible for the failure of starter cultures. The evidence available suggests that phage in streptococcal cultures commonly originates from the organisms themselves (Whitehead & Hunter(7)), but a complete proof of that theory is lacking at present. It is quite certain, however, to our mind that starter cultures do suffer failure due to phage action under conditions which preclude all possibility of immediate contamination from outside sources.

When this stage in starter investigations was reached it seemed to us that the problem could be stated as follows: Bacteriophage is associated with, or can originate from, the organisms in most cultures of lactic streptococci. Is it possible to define conditions for daily subculture under which the phage is unable to make its appearance?[1]

[1] The difference between the appearance of phage and the development of phage in a culture should be clearly understood. Added phage can develop under any conditions which permit growth of the substrate organism. The spontaneous appearance of phage in a streptococcal culture occurs only at certain times and under special circumstances (e.g. in aerated milk). We are concerned in this paper mainly with the spontaneous appearance of phage.

The following paper gives an account of some attempts to discover the requisite conditions, and indicates a technique which has enabled us to maintain cultures of lactic streptococci in a state of constant activity over a long period of time. It is to be understood that unless otherwise specified the work reported in this paper was carried out with single strain cultures (in the main with culture HP, which of all our cultures has had the widest use in cheese manufacture in New Zealand). In the course of our work on starters we have come to the conclusion that little purpose is served by an investigation of unknown mixtures of streptococci. Even if the use of mixtures ultimately proves desirable for cheese manufacture much fundamental work is necessary on the properties of single strains, and any mixtures finally decided upon must have a known composition and be reproducible at will. Most of the mixed cheese starters in use to-day suffer extensive changes in their flora (and hence in their acid-producing activity) within a few months, and the essential characteristics of any given culture can never be reproduced. The conclusions reached as a result of experiments carried out on such starters are not likely to have a general application, and to that extent the effort expended is largely wasted.

CIRCUMSTANCES UNDER WHICH THE BACTERIOPHAGE MAKES ITS APPEARANCE

It is desirable first of all to describe the exact conditions under which, until recently, starters were propagated in New Zealand cheese factories. The following procedure had been gradually developed over a period of years to replace the older rough and ready methods. The details of the process and the care exercised in carrying it out naturally varied considerably from factory to factory; but the important point to be noted is that even when the procedure was followed carefully (and sometimes elaborated in certain particulars) by persons trained in aseptic technique, failures were relatively common. There was ample evidence, in other words, that the exercise of a careful bacteriological technique did not eliminate failures of the starter cultures.

Mother cultures

Mother cultures were maintained in conical flasks; about 600 ml. of milk were poured into a litre conical flask, the mouth of which was covered with a small cap of aluminium or stainless steel. The flask was heated in flowing steam for 45–60 min. and thereafter was cooled to a temperature of 21–24° C. The flask was then inoculated from the previous culture (now clotted and with an acidity of 0·7–1 % lactic acid) by means of a pipette which had been sterilized either in the steaming vessel or in a metal cylinder by dry heat; alternatively, the inoculation was carried out by pouring a little culture directly from flask to flask. The size of inoculum varied from one drop upwards; it rarely exceeded 1 ml. The inoculated culture was maintained at 21–24° C.,

either in a special incubator or by partial immersion in a large bulk of water at the correct temperature.

Bulk cultures

The bulk culture for use in the cheese vats was prepared by "sterilizing" milk in quantities of 15–20 gal. in cylindrical cans. The cans were partially immersed in a rectangular tub of water which was raised almost to boiling-point by steam injection; the milk was maintained at a temperature ranging from 88 to 96° C. for at least 1 hr. (usually much longer). Then it was cooled to 21–24° C., inoculated, and maintained overnight at approximately 22° C. by occasional adjustment of the water temperature. In some factories large pieces of equipment of 150–200 gal. capacity were used for starter preparation. The treatment of the milk was very similar to that already described, except that usually some mechanical arrangement for continuous stirring of the milk during heating and cooling was fitted to the apparatus. Inoculation of the bulk starter milk was carried out either by direct pouring from the neck of the mother culture flask or by measuring out the culture in a sterilized dipper. The size of inoculum varied considerably; 120 ml. per 15 gal. of milk would be an average amount. There had been a tendency in recent years to reduce the size of inoculum in both bulk and mother cultures as far as possible in the belief (supported by most text-book authorities) that over-ripening was harmful and that it accounted for many of the failures experienced.

Characteristics of failures of single strain cultures

The phenomenon of failure due to phage exhibited several different aspects under factory conditions. Given a perfect technique in the management of the starter, failure was extremely rare in the mother culture. The most obvious type of failure in the bulk starter was a failure to clot within 24 hr. Phage developed apparently spontaneously in the starter cans, and the streptococci were lysed at an early stage in their growth; the acidity of the "thin" culture might have any value between 0·18 and 0·5 % calculated as lactic acid. A less obvious type of failure was one in which the bulk-starter culture had a normal appearance and acidity. Vats in which such a culture was used might show a cessation in acid development at almost any stage in the cheese-making process. Examination of such a bulk starter in the laboratory would show that phage was present; it had developed rather too late to prevent the clotting of the culture, but it was there in sufficient quantity to destroy the streptococci in the next generation (i.e. in the vat). If the phage was present in very small amount there might be no other sign of its presence than a slight slowing in acid development towards the end of the cheese-making process; with larger amounts the process might be halted almost at its start. Until it was made clear that bacteriophage action was the basic phenomenon these various types of failure and partial failure had been very confusing.

In the laboratory, investigation of the factors which promote appearance of phage in single-strain cultures led to a slight clarification of the position;

but the problem was complicated by the fact that even under regular conditions of subculture in tubes or in flasks of sterile milk cultures were not constantly susceptible to spontaneous phage development. This phenomenon of periodic sensitivity has already been described by Whitehead & Cox(2). It rendered (and still renders) the investigation of the phage problem very difficult because cultures could not be induced to phage at will; the phage appeared under the appropriate growth conditions only when a culture was "sensitive", a state which seemed to occur sometimes only at long intervals. As a result of work carried out over several years, however, the following position had been reached.

When a culture is in the "sensitive" state the following factors promote the appearance of phage:

(*a*) Aeration of the culture milk immediately prior to inoculation. This has already been described by Whitehead & Cox(2). The phenomenon must be distinguished from that observed by Virtanen & Tammisto(8) (and referred to later), who found that aeration of milk *during the pasteurizing process* tended to preserve starter cultures from failures which resembled phage failures. Virtanen's treatment might help to eliminate dissolved gases from the milk; it would not incorporate atmospheric oxygen in the milk as aeration immediately before inoculation would do.

(*b*) Use of a large volume of milk as in the preparation of bulk cultures for commercial use. The underlying reason for any difference in result between large and small cultures treated in almost identical manner is difficult to explain. In some of our experiments starter cultures were prepared in cylindrical glass vessels of 250–1000 ml. capacity, plugged with cotton-wool; the cultures never failed in vessels of the smaller size and very rarely in the larger size. Attempts to prepare larger amounts of the same culture in cylindrical cans of 10 gal. capacity plugged with a large mass of cotton-wool failed on every occasion.

(*c*) Use of milk which has been allowed to stand for several days between the time of sterilization and the time of inoculation. In one series of experiments where inoculated tubes of sterile milk were incubated in a water-bath at 37° C., several cases of spontaneous phage development occurred. It was eventually shown that this phenomenon happened with sterilized milk 4 or 5 days old, never with freshly steamed milk.

In addition to the above factors there was a suggestion that failures occurred much more readily in some milk supplies than in others. On one or two occasions it seemed reasonably certain that a sensitive culture would suffer failure due to phage action when milk from a certain source was aerated, whereas in another milk supply it would grow normally even when aeration was practised. Only very recently, however, we encountered a milk supply in which there existed some factor which undoubtedly stimulated phage appearance. This must be the subject of future investigation.

Empirical solution of the problem of phage failure

As has been indicated, laboratory findings and experience in commercial factories had both led to the conclusion that the genesis of the trouble lay within the streptococcal cultures themselves. Innumerable variations in the conditions of growth of the organisms were tried with no success until a statement made by Davis (9) (in quite another connexion) finally gave an idea which led to an empirical solution of the problem. Davis says (speaking of *L. delbruckii*) "washing of the cells also greatly reduces the respiratory activity which appears to be activated by substances attached to the surface of the cell or perhaps easily diffusible. Weakened cultures may develop an "oxygen sensitivity". Although *Str. Cremoris* cannot be likened to *L. delbruckii* in its respiration activity, it seemed possible that the relatively light inoculation which had hitherto been used in cheese starter cultures (in New Zealand) might result in a washing of the bacterial cells in the new batch of milk. The cells would thus be exposed to rather unfavourable surroundings particularly with regard to Redox potential, and this might be connected with the appearance of phage. Would a more massive inoculum result in a protective action due either to less complete exposure of the individual cells to the fresh medium or to mere presence of larger amounts of products of growth from the previous culture?

A trial was made by increasing the inoculum used in a 600 ml. mother culture from a few drops to 10 ml. and in a bulk starter culture from about 0·2 to 1 % (i.e. 1½ pt. to 20 gal.); no other modification was made. The effect was remarkable. The acid-producing power of a single-strain culture seemed to be stabilized immediately. Sensitive single-strain cultures in use at the Institute Experimental Factory had, under the original system of maintenance, manifested an unsteadiness in performance from time to time even when complete failure did not occur. From the time of application of the heavy inoculation system the cultures began to give absolutely regular results (so far as acid production was concerned) from day to day with only a gradual alteration in performance as the milk composition altered due to progress of lactation period or major alterations in weather. Experience over an 18-month period has shown that at the Institute the system eliminates the typical phage failures which formerly occurred.

Limits of heavy inoculation

The selection of a 1⅔ % inoculation for mother cultures and a 1 % inoculum for bulk cultures was purely arbitrary. The most obvious effect of the heavy inoculation on the cultures was to cause more rapid clotting and an "over-ripening" during the period of incubation. Cultures maintained under the new system showed on removal from the incubator a slightly contracted clot

and a thin layer of clear whey on top of the culture. In view of the old-established idea that "over-ripening" had a deleterious effect on starter cultures (apparently at variance with the present finding that a technique involving "over-ripening" eliminates phage failures), it was desirable to find out how far the inoculum could be increased without causing damage in the case of single-strain cultures. The general effect of prolonged incubation and high incubation temperature was also investigated.

It was found in the first place that single-strain cultures tended to reach a very definite and constant final acidity no matter what the size of inoculum and time of incubation at 22° C. Increased rapidity of growth due to heavy inoculation naturally caused a more rapid clotting of the culture. For instance, a lightly inoculated culture (up to 0·5 to 600 ml.) incubated at 22° C. showed no appreciable increase in acidity for 8 or 9 hr.; clotting occurred only after 12 hr. or more. An increase in the size of inoculum to 10 ml. gave a measurable increase in acidity after 3–4 hr. and clotting occurred after about 10 hr. There was no significant difference in acidity between lightly and heavily inoculated cultures after 24 hr. This suggests that the effect of heavy inoculation in eliminating phage appearance must be due to conditions operating during the very early stages of growth (cf. later discussion). Increase in the size of inoculum up to 10 % had no appreciable effect on the final acidity reached by a culture, nor did the culture appear to show any permanent damage under such daily treatment. The only significant effect caused by exceedingly heavy inoculation or by extension of the period of incubation (up to 3 days at 22° C. was tried) was to render the culture somewhat sluggish in acid production in its next transfer. A bulk starter kept for 2 days and then used in the cheese vat in the usual proportion of 1–1½ % was subnormal to a slight extent in its activity. The effect was not, however, permanent; growth for one generation under more normal conditions (1 % inoculum and 24 hr. at 21–24° C.) always restored the original activity of the culture. The effect of "over-ripening" therefore was merely to stifle temporarily the organisms in a culture; no permanent damage was done over very wide limits of variation of size of inoculum and time of incubation. It was concluded finally that the conditions originally specified arbitrarily were not near any danger zone so far as the single-strain cultures in use were concerned.

The limits of incubation temperature permissible have not yet been fully investigated. The indications are that at some temperature between 30 and 37° C. an effect comes into play which leads to a permanent irreversible decrease in the activity of the streptococci. A temperature of 24° C. appears to us to be the optimum under commercial conditions.

The essential effect of heavy inoculation

The effect of heavy inoculation in the maintenance of starter cultures was an empirical result reached from a very slender theoretical basis. To complete the picture it was necessary to get some idea of the essential mechanism. In

the first place the suggestion arose (in spite of evidence to the contrary already reported by Whitehead & Hunter (7)) that traces of phage were always present in our cultures, and that heavy inoculation merely suppressed the phage by balancing conditions in favour of the streptococci. It is impossible to prove that phage does not cling to organisms throughout many processes designed to purify cultures, and therefore many "pure" cultures may contain traces of phage. The following experiment was designed to test the effect of "heavy" versus "light" inoculation on the development of a minimal amount of phage added to a culture.

A phage for culture HP was diluted in series in sterile saline and drops from the successive dilutions were added to two series of sterile milk tubes (10 ml.). Thus the two series of milk tubes now contained milk with progressively decreasing traces of phage. The two series of tubes were inoculated with culture HP, one with a light inoculation by means of a straight wire, the other with five drops of culture from a sterile pipette. Incubation was at 22° C. for about 20 hr. The tubes in which clotting occurred (i.e. in which the phage had not lysed the organisms) were subcultured daily in sterile milk, heavily or lightly according to the series. No more phage was added to the tubes subsequent to the first addition which was designed to simulate contamination of a culture with extraneous phage. The results of the experiment are given in Table I.

Table I. *Comparison of heavy and light inoculation in presence of traces of phage*

	Logs of concentration of phage in original culture tubes													
	Heavy inoculation							Light inoculation						
	−11	−12	−13	−14	−15	−16	−17	−11	−12	−13	−14	−15	−16	−17
1st culture	C	NC	C	C	C	C	C	NC	NC	NC	NC	C	C	C
1st transfer	NC	—	NC	NC	C	C	C	—	—	—	—	NC	C	C
2nd ,,	—	—	—	—	NC	NC	C	—	—	—	—	—	C	C
3rd ,,	—	—	—	—	—	—	C	—	—	—	—	—	C	C

C = clot; NC = no clot (complete lysis of streptococci)

It will be observed that the only specific effect of heavy inoculation was to preserve some of the cultures from phage action for one generation longer than was the case with light inoculation. Ultimately there was no essential difference in result between the lightly and heavily inoculated tubes; when more than a certain minimal amount of phage was present originally it increased with each subculture until the organisms suffered lysis. The experiment was repeated several times with other organisms and their appropriate phages, always with the same result. It follows, therefore, that the influence of a heavy inoculation cannot be ascribed to a suppressing effect on phage present in a culture, because where a starter culture contains contaminant phage it suffers phage failure just as surely under heavy inoculation treatment as under light inoculation treatment. The results of the above experiment tend to support still further the theory that the phages encountered in

streptococcal cultures maintained under a light inoculation were produced by the organisms themselves or were present in some special form of association with the organism; otherwise it is difficult to see how the mere change to a heavier inoculation could have had so marked an effect.

The above negative aspect of the problem having been discussed, it remained to attempt to define the significant effect of heavy inoculation on the growth conditions of streptococci, which rendered the organisms so much less likely to produce or liberate phage. The impossibility at present of stimulating with certainty the appearance of phage even under the most favourable conditions for its production makes the testing out of any theory a matter of time. A consideration of the whole set of phenomena led us to the theory that the essential difference between heavy and light inoculation lies in their respective influences on the lag period which follows the subculture of organisms into a fresh batch of medium. Aeration of the milk, ageing of the milk and light inoculation itself can all be shown to give a prolonged lag period. Their relative effects are illustrated in the following experiment in which methylene blue reduction times were used to indicate rates of growth of the organisms.

Ninety tubes each containing 10 ml. of skim milk were plugged with wool and steamed on three successive days. Thirty of the tubes were steamed again on the fourth day, while the remainder were stored in the media cupboard. After an interval of 5 days the remaining sixty tubes were steamed a fourth time. After these tubes had cooled, the milk in thirty of them was aerated; it was poured from the tubes into a sterilized beaker, and from there back and forth about five times into another sterilized beaker. Then it was replaced in the tubes. Every care was taken to avoid contamination during this process. The three series of milks (aged, freshly steamed and aerated) were then divided into three groups of ten each, which were incubated at 20, 30 and 37° C. respectively, and each group of ten was divided into sets of five. Five tubes in each group were inoculated with a small drop (about 0·02 ml.) of culture HP from a sterile dropping pipette and the other five with five drops from a 1 ml. sterile pipette (about 0·2 ml.). Sterile methylene blue was added to all the tubes and the times required for reduction were observed. The five tubes in each set gave almost identical results. In the following table the average reduction time for each set of five is given.

Table II. *Methylene blue reduction time (in hours) in one batch of milk treated in various ways*

Temp. ° C.	Inoc.	Aged milk	Freshly steamed milk	Aerated milk
37	Heavy	2	½	1¼
	Light	5	2	3¼
30	Heavy	2	½	1¼
	Light	4½	2	3
20	Heavy	3¼	2	2½
	Light	7½	5	6

It will be observed that light inoculation, aeration of the milk, and ageing of the milk, are all factors which tend to increase the lag period which precedes active growth in a streptococcal culture. Other experiments showed that no further effect is produced by ageing for more than five days. It is reasonably certain that the effect of ageing is exerted through the atmospheric oxygen which "soaks" into the milk on prolonged standing at room temperature and that therefore aeration and ageing represent the same phenomenon. Unfortunately, it has not yet been possible to carry out an experiment of the above type with a culture in its most sensitive state when it would be expected that a proportion of the aerated and aged milks would show spontaneous phaging as has been observed in scattered instances from time to time in the past. The complete proof of a connexion between lag period and phaging tendency is therefore lacking. However, at least three factors which increase the lag period, light inoculation, aeration of milk and ageing of milk were observed over a period of time to increase the tendency for phage to appear spontaneously in cultures. (It has even been observed in several instances that failure due to phage appearance may occur in a cheese vat which has been deliberately inoculated so lightly with starter ($\frac{1}{4}$ % instead of 1 %) that acid development in the "cooking" period is very slow.) The fourth factor mentioned, the bulk of the culture prepared, would not appear to exert an influence on lag period, especially if the proportion of inoculum were maintained constant; but a re-examination of this question is necessary. Dissolved oxygen is more difficult to remove from a large bulk of milk than from a small one, and it is possible that with the usual times of heating of bulk starter milk there is an incomplete removal of gases and hence a delayed growth of the streptococci. Very recently, when an instance of starter failure due to a milk difference or abnormality was encountered in a commercial factory, a trial of a method of treatment similar to that recommended by Virtanen & Tammisto(8) was made. The milk used for the starter culture was heated to about 90° C. and was then poured about and agitated as thoroughly as possible, with the idea of removing gases and volatile substances. Subsequently it was sterilized in the usual way, cooled and inoculated. There was evidence of some improvement when this method was used, but failures were not completely eliminated. This particular milk abnormality remains unexplained at present.

On the basis of the theory that any factor which delays growth of a lactic *Streptococcus* increases the tendency for phage appearance, we have laid emphasis in commercial practice on the desirability of encouraging the rapid initiation of growth in starter cultures and of avoiding any practice which tends to increase the lag period which must necessarily occur when cultures are transferred into fresh batches of milk. Thus the use of freshly heated milk, heavily inoculated (1–1$\frac{1}{2}$ %) and incubated at 24° C. is recommended. The necessity for an aseptic technique is obvious. The precise effect of heavy inoculation on the lag period and on the suppression of phage is of course unexplained. It is impossible as yet to say whether the statement made

by Davis (our original starting-point) has any actual bearing on the phenomenon.

Extraneous contamination with bacteriophage

The application of the system of heavy inoculation in commercial practice was attended by unexpected difficulties. Failures of single strain starters were frequently reported in both mother and bulk cultures, even where it was claimed that the recommended technique was being practised carefully. Much work in the field was carried out; workers from the Institute remained at factories to run the starter for periods up to a month. It was finally proved beyond reasonable doubt that in the majority of cases the failures were due to faulty technique which led eventually to contamination of the cultures with extraneous phage. In view, however, of the strain-specific character of most of the phages so far isolated it was rather difficult to imagine where the phage originated, especially in cases where a culture had not previously suffered phage action at that particular factory. A search for the origin of the contamination led to the rather unexpected discovery that phage active against the homologous streptococci was often present in the whey in a normally working vat in which a particular single strain was in use. The phage could be detected in the whey during the later stages of the cooking period, and it increased progressively until it was in very strong concentration in the whey at salting. Coincidently the acid development in the curd proceeded normally. This phenomenon has still to be investigated more fully. At first sight it would appear that contamination of the vat with phage by chance and then a "carry-over" infection from day to day would explain the sequence of events. It can be shown, however, that where any appreciable quantity of phage finds its way into a vat of milk the acid development in the cheese curd will be inhibited to a greater or less extent depending on the extent of phage contamination. Furthermore it was shown that with sterilized milk treated with calcium chloride and made into curd under aseptic conditions (except for non-sterile rennet) appearance of phage in the whey could often be demonstrated. Therefore in spite of the fact that a cheese vat is open to contamination by any phage present in its surroundings, it did seem as though the phage had appeared spontaneously and that the conditions in cheese whey in contradistinction to those in the curd were conducive to its appearance. The phenomenon will be investigated under laboratory conditions in the hope that a means for the production of phage at will may be evolved. In the cheese factory it has already proved possible to isolate phage for one of the organisms in a mixed starter from the whey in a vat in which the starter was used. On the other hand, some single strains have not yet yielded a phage under the conditions obtaining in a cheese vat. In any case the demonstration of phage in normally working vats provided a ready explanation for the frequency of failures in factories where the technique of starter maintenance was not free from flaws.

Discussion

The theory that a prolonged lag in the initiation of rapid growth in a culture of streptococci favours the spontaneous development of bacteriophage in the culture is not very complete and satisfying. What brings about the lag period? Do the individual organisms remain dormant for a longer time in the lightly inoculated culture or does the presence of more organisms in the heavily inoculated culture merely mean that the culture as a whole is for a shorter time under unfavourable growth conditions, due to the fact that the organisms (owing merely to their greater number) can more quickly produce acid and attain a certain oxidation-reduction potential? It would be easy to develop hypotheses involving oxidation-reduction potential, respiratory systems and so forth to explain the effect of aeration on the lag period but it would not be profitable to do so at present, firstly because little or no evidential backing is available and secondly because the final phenomenon, the apparently spontaneous development of bacteriophage, is likely to remain inexplicable until phage can be made to appear at will and more is known of its nature. We have shown that certain methods of treating a starter culture cause a prolonged growth lag, and have suggested that this lag results in the development of phage from the organism. It seems to us that it is more profitable to collect more facts at present, than to build theories in an attempt to bridge the obvious gaps between cause and effect.

From the practical cheese-making viewpoint it seems as though the methods outlined here give the means for a more accurate control over starter activity than has hitherto been possible. As this paper was about to go to press, an instance was encountered of a milk supply in which failures occurred even with heavy inoculation and an aseptic technique. This is the first such case encountered by us in two years of trials in the field. It remains to be seen how often such milks occur and whether any further modification of technique will eliminate phage failures in them. There are milks, as has already been indicated, which appear to give trouble more readily than others where a light inoculation is practised. Heavy inoculation, however, usually overcomes this particular effect. A big difficulty in practice is the training of the factory worker in an aseptic technique which will enable him to maintain a culture free from contamination by the phage which is present in his surroundings.

The question of whether a single-strain streptococcal culture fulfils all the requirements of a cheese starter is an entirely separate one. If mixtures of organisms are finally found necessary, precautions against phage appearance and phage contamination will still be needed. Even where mixtures of unknown composition are used our most recent findings show that some of the trouble often experienced is very probably due to phage action. It is evident that even now the whole subject of starters for Cheddar cheese-making has merely been opened up. The work on bacteriophage and the knowledge gained on the

factors promoting its appearance seem to provide a very promising avenue for attack on the problem; the results obtained in rather an empirical fashion can in the meantime be put into practice with profit.

SUMMARY

Single-strain cultures of streptococci used as "starters" in Cheddar cheese manufacture in New Zealand frequently suffered failure due to the apparently spontaneous appearance of bacteriophage. The trouble was cured at the Institute experimental factory by increasing the inoculum used in the starter culture from about 0·2 % to 1·0–1·6 %. The culture then showed a regular activity in the cheese vat from day to day for long periods of time. Field trials during the past two years have indicated that single strain cultures can be maintained for long periods also in commercial factories if the correct technique is practised.

The theory is postulated that the effect of heavy inoculation in eliminating the spontaneous appearance of bacteriophage is due to the more rapid growth of the streptococci when they are transferred to each new batch of milk, i.e. to the shortening of the lag period. Bacteriophage tends to appear whenever the lag period is prolonged as a result of light inoculation, low incubation temperature, and aeration or ageing of the milk medium. Until more is known of the nature of phage it is not possible to suggest why a prolonged lag period in the initiation of growth should favour the appearance of phage.

Under commercial conditions failures of starters occur from time to time due to faulty technique which permits the entry into cultures of extraneous phage. The source of the contamination is most probably the whey from cheese vats, since it has been shown that phage commonly occurs in the whey even in vats where acid develops quite normally within the the cheese curd. A recent case of failure, which appears to be due to some milk abnormality, indicates that there are some milk supplies in which the heavy inoculation technique described in this paper is not completely effective in preventing the appearance of bacteriophage.

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REFERENCES

(1) Whitehead & Cox (1935). *N.Z. J. Agric.* **50**, 107.
(2) —— —— (1934). *J. Dairy Res.* **5**, 197.
(3) —— —— (1935). *N.Z. J. Sci. Tech.* **16**, 319.
(4) —— —— (1936). *J. Dairy Res.* **7**, 55.
(5) Nelson & Hammer (1937). *J. Dairy Sci.* **20**, 444.
(6) Mazé (1937). *C.R. Soc. Biol., Paris*, **125**, 412.
(7) Whitehead & Hunter (1937). *J. Path. Bact.* **44**, 337.
(8) Virtanen & Tammisto (1936). Summary of work in Hansen's *Dairy Bulletin*, Jan.–Feb. p. 8.
(9) Davis (1937). *Proc. Int. Congr. Microbiol.* p. 197.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION A. PHYSIOLOGY OF DAIRY CATTLE[1]

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I. REPRODUCTION AND LACTATION

ALTHOUGH research work on the physiology of reproduction in dairy cattle does not appear to have made any outstanding advance during the past two years, some slight but valuable progress is reported in a few of the more important branches of the subject. Non-pathological sterility, one of the greatest problems for the dairy farmer, continues to be studied in detail. Its importance could not be better gauged than by reference to the published account of a lecture by Craig(1) in which this recognized authority presents a concise and clear account of the problem as it exists to-day. The value of the account is enhanced by the subsequent discussion in which various points are raised by questioners possessing first-hand knowledge of the practical difficulties involved. Lagerlöf(2) has published a treatise on sterility which opens with sections devoted to its etiology, genetics and hygiene. He then passes to the main part of the paper which deals with sterility in the bull and in which he stresses that a study of the morphological characters of the spermatozoa is more important for detecting permanent sterility in bulls than their actual number and motility, because active motility does not necessarily imply fertility and in old bulls the number may be perfectly normal although fertility is declining. The various types of spermatozoa are described and illustrated and it is explained that the number of spermatozoa deviating morphologically from the normal is a point of importance. Thus for the 100 normal bulls examined this number was found to be 117 per 1000, but with bulls having defective development of the testes, those in which sterility set in suddenly and in aged bulls the corresponding number was well over 300. In this connexion it may be mentioned that the development of the testes and the scrotum of the ram, bull and boar has been studied in detail by Phillips & Andrews(3) with the object of collecting information which might lead to the better breeding, management and feeding of young males.

The influence of diet on reproduction has been investigated by Arnold *et al.*(4) particularly with regard to mineral requirements and by Meigs & Converse(5) with reference to vitamin A. Arnold and his colleagues find that under natural conditions in Florida, salt sickness retards reproduction and that its results can be overcome by the addition of ammonium citrate and copper sulphate to the drinking water or by free access to bone meal and certain mineral mixtures. Calves from heifers suffering from this deficiency had an average weight of only 39 lb. as compared with 54 lb. for those from normal heifers, and the lighter calves were more delicate and more prone to ailments of various kinds. Copper and perhaps iron appear to be essential in the diet for normal reproduction. Meigs and Converse confirm the results of other workers, mentioned in the previous review, who have stressed the necessity for vitamin A or carotene for normal healthy reproduction in cows. They have found that with rations poor in carotene there is a large proportion of premature births,

[1] Owing to the rapid growth in the number of papers falling for review under the general heading of "Physiology of Dairy Cattle," it has been decided to transfer the section entitled "Genetics of Dairy Cattle" to a separate review which will appear in the next issue of the *Journal*.

still births and of weak and blind calves. Thus with 26 parts of carotene per 100,000 of hay, the percentage of normal births was 91, with only 8 parts it was reduced to 29 and with 3 parts no normal birth took place. Reproduction on the poorer rations was greatly improved by supplementing them with vitamin A or carotene, while diets causing only 29% of normal births did not have such a harmful effect if the cows were fed on them for not longer than 6 months after a previous period of good fresh pasture. In a later paper(6) the same authors conclude that for normal calving, cows should receive 80–100 mg. carotene daily and that if this value falls as low as 60 mg. a considerable proportion of dead calves must be expected. The loss of 84 newborn calves probably resulting from some dietary deficiency is described by Sampson *et al.*(7). The calves appeared normal at birth, but at about 6 hr. old they were too weak to stand and suffered from profuse diarrhoea. They usually died within the next 24 hr. A change in the diet of the cows caused a marked improvement. Two papers dealing with dietary factors and sterility in rats may be of interest here in view of their possible application to dairy cattle. They are published by Pearson *et al.*(8,9) who find that when gliadin is fed as the main source of protein, cessation of oestrus occurs, and that the cessation can be cured by the addition of lysine to the diet containing gliadin. With casein as the source of protein, added lysine only partially restores oestrus, complete normality occurring only when both lysine and gliadin are added together. The authors conclude that for the prevention of sterility in rats there are two important dietary factors of this type, and they point out that no permanent sterility results from these deficient diets as long as the necessary factors are subsequently provided. It would be of interest to establish whether these findings are in any way applicable to dairy cows.

The influence of environment on reproduction in Zebu cattle has been studied by Anderson(10,11) who found that successful matings tended to occur more readily at certain seasons of the year. This might have been due to some environmental factor particularly favourable to the occurrence and duration of oestrus which in these cattle is extremely short, lasting only for 0·2–2·9 hr. as compared with 6–30 hr. in British cattle. He found that rainfall, temperature and seasonal variations in pasture did not affect the periodicity and duration of oestrus but that there might be some correlation between sunshine and the length of duration. After much work the author concludes that this line of investigation is unlikely to lead to useful results in the study of sterility.

An analysis of variations in the sexual cycle and some of its component phases with special reference to cattle is published by Chapman & Casida(12). It is much too detailed for adequate treatment in a brief review, but it may be pointed out that it deals with records kept for 10 successive years and compares the data for cows with those for certain other species. The results are treated statistically under such headings as "Statistics of component phases of the sexual cycle" and "Frequency distribution of the periods from parturition to first subsequent oestrus in normal and abnormal cattle", etc. One interesting point made by Chapman and Casida which confirms an observation of Anderson's(11) is that they could find no definite effect of seasonal changes on the duration of oestrus in cattle and sheep nor on the cycle lengths in cattle. Sterility in cows in South Africa under the influence of such factors as dry rations, lack of exercise and lack of sunlight has been investigated by Quinlan & Roux(13).

Belief in the functional superiority of the right ovary as accounting for the greater number of pregnancies occurring in the right uterine horn, has long been generally held without the existence of much evidence in the literature to support it. A recent publication by Reece & Turner(14), however, confirms this belief, for amongst other evidence presented, these authors state that the largest follicle was

found in the right ovary in 75% of 136 immature heifers and that in 64% of 36 pregnancies the corpus luteum was found there also. It was further observed that the first ovulation occurred in the same gland.

A subject of considerable importance in reproduction is the diagnosis of pregnancy in animals. Two biological methods are at present available. One depends on the presence of gonad stimulating hormones in the urine or in the blood serum, and the other on that of oestrone in the urine. Anderson(15) discusses this subject and finds that in the mare pregnancy can be diagnosed at latest by the 60th–65th day, if not before, by the oestrone of the urine, the content of this hormone increasing as the period of gestation proceeds. In the cow and goat, however, no measurable amount of antepituitary hormone occurs either in the blood or the urine and even the amount of oestrone in the urine is so small and variable, particularly in the early stages of pregnancy, that a clinical examination can be made before a biological diagnosis is possible. Anderson therefore concludes that biological diagnosis of pregnancy in dairy cattle is not yet feasible. Recent researches on hormones are dealt with in detail in the next section but it may be well to mention here that a brief review on the subject in connexion with pregnancy has been published by Miller(16), and that Turner(21) has suggested lines of research for the study of endocrinology in relation to inheritance which he considers might ultimately lead to better results in the breeding of cattle with high producing ability.

Other topics which have been investigated during the past two years are the cholesterol metabolism of the pregnant cow and the foetus by Sato(17), foetal respiration by Roos & Romijn(18), and the inheritance of producing ability and other properties in dairy cattle by Dickerson(19) and Labes(20).

Hormones

It is no exaggeration to say that during the past few years more work has been published on the rôle of hormones in the initiation, maintenance and inhibition of lactation than on any other aspect of the subject. This is obviously desirable, for it is clear even now that a complete understanding of the complex processes involved in milk secretion will only be obtained when the part played by each individual hormone has been thoroughly worked out. In fact so great has been the volume of papers produced under this heading during the past two years that it has not been found possible to refer to each one separately, but it is hoped that mention has at least been made of the majority of those which may have a direct bearing on the subject of reproduction and lactation.

One great advantage enjoyed by workers on hormones is that the functions of the endocrine system seem to be fundamentally the same for most mammals. It is therefore possible to carry out much work on small animals such as the guinea-pig and the rat with considerable confidence that what holds for them will also probably apply, with no more than slight modification, to other animals such as the cow and the goat. Consequently if the present position is to be understood, it is essential that reference should be made in considerable detail to the work on these smaller animals.

Many of the investigations can be divided for convenience into two sections, those which deal with development and growth of the mammary gland on the one hand and those which are concerned with the initiation and maintenance of lactation on the other. When the previous review was prepared, there were two conflicting views with regard to the precise action of the ovarian and pituitary hormones on mammary development. Several workers such as Nelson(22) and Asdell & Seidenstein(23) contributed to the theory that the ovarian hormones cause growth and development of the mammae and inhibition of lactation even in the absence of the

hypophysis, while the presence of pituitary hormones is essential only for the actual process of lactation itself. On the other hand, publications by Selye *et al.*(24, 25, 26), Reece *et al.*(27) and Lyons & Pencharz(28) suggested that oestrin and progesterone, either separately or together, are quite without effect on the mammae of the hypophysectomized animal, and that these hormones are largely dependent on the pituitary for their full activity.

The essential nature of the pituitary hormones in this connexion now seems to have been confirmed particularly by the work of Gomez, Turner and their colleagues(29, 35, 36, 38, 39), who have carried out extensive investigations on hypophysectomy and replacement therapy in relation to the growth and secretory activity of the mammary gland and have published a concise and comprehensive summary of their experiments and conclusions in a special bulletin(40). They explain the divergence of views on the essential nature of the hypophysis for the action of the oestrogens as being due to the possibility that in some instances where hypophysectomy was carried out fragments of the gland may still have remained in the animal and may have supplied sufficient of the pituitary hormones to give rise to the erroneous conclusions—an explanation which seems most feasible when the difficulties of the operation are considered. Experiments by Gomez & Turner(35) have shown that mammary development can be stimulated in hypophysectomized guinea-pigs by implantation of pituitaries from rats which have previously been given oestrogenic hormones, but that pituitaries from rats which have not been treated with oestrin fail to show the same action. It is therefore suggested that the pituitary is influenced by oestrogenic hormones to secrete a substance, other than prolactin, which is capable of inducing mammary development and of inhibiting lactation. These authors(38) have consequently inferred the presence of a mammary gland growth-promoting factor which is quite distinct from the lactogenic hormone, prolactin, and which they term the "mammogenic hormone". This theory was confirmed by the fact that pituitary extracts from pregnant cattle caused growth of the mammae when injected into castrated female rabbits and rats, whereas when similar preparations from non-pregnant cattle were used there was no such effect, although the lactogenic hormone prolactin was present in both cases.

With regard to the initiation and maintenance of lactation Gomez & Turner (30, 32, 33, 34, 36, 37) find that injections of purified prolactin preparations (as distinct from crude pituitary extracts) into various animals such as rats, rabbits, guinea-pigs, cats, etc., in which lactation had ceased due to hypophysectomy, did not reinitiate milk secretion, and this led them to emphasize the importance of other hormones not only from the pituitary but also from glands such as the thyroid and the adrenal cortex. They even found that in pigeons(31) which are used for the estimation of prolactin, this hormone in a purified condition was incapable of causing crop proliferation except in the presence of the pituitary. They(32) therefore injected adrenal cortical extract and glucose as well as prolactin into hypophysectomized animals and found that although these substances had no action separately, together they were able to reinstate and maintain lactation although they did not prevent the involution of the mammary gland. In later experiments(33) they substituted the adrenotropic hormone of the pituitary itself for the adrenal cortical extract and concluded that the reason for cessation of lactation on removal of the pituitary is the absence of at least three hormones, the lactogenic, the adenotropic (eschatin) and one influencing carbohydrate metabolism. Nelson & Gaunt(41), working in the same field, have recently produced results confirming the fact that prolactin will not reinitiate lactation in hypophysectomized guinea-pigs after 8 days' treatment, while either crude pituitary alone or prolactin with cortical hormone will induce it. This again showed that the adenotropic hormone is essential, a fact which obtained further confirmation by

adrenalectomy. As adrenal deficiency is known to be alleviated by salt therapy, these authors studied the effect of administering sodium chloride to hypophysectomized guinea-pigs. They found that salt therapy accompanying prolactin injections induced lactation, and thus again confirmed the necessity for the adrenotropic hormone. Hall *et al.*(42) have surveyed quantitatively the administration of large doses of the adrenal cortical hormone preparations to sexually mature rats before mating, during pregnancy and during lactation, the diet of the animals being made deficient in minerals in the direction which the hormone might be expected to overcome. These authors claim that this hormone tends to cause a marked increase in the incidence of pregnancy and the number of foetuses, but that on a normal diet it has little effect during actual pregnancy or lactation. When, however, the diet was deficient in sodium, the hormone was found to have a beneficial effect. When it is considered that these results were obtained for intact and not hypophysectomized or adrenalectomized animals it will be seen that they do not in any way conflict with those of other workers, for probably it is only in the absence of the pituitary or of the adrenals that the full necessity for adrenotropic or adrenal cortical hormones may be observed. A later paper by Pencharz & Lyons(43) shows that, while prolactin and the adrenotropic hormone together induce milk secretion in hypophysectomized guinea-pigs, the actual yield of milk is not sufficient to sustain the young. They therefore suggest that the action of the pituitary in lactation cannot entirely be replaced by these two hormones, a suggestion which is again in complete agreement with the results and theories of others.

For a brief and yet detailed account of the whole problem as it appears to-day, the reader could not do better than study the general conclusions arrived at by Gomez & Turner on both mammary development and the lactation process itself as presented in their recently published bulletin(40), which is excellently illustrated by diagrams and microphotographs and is well worthy of more detailed treatment than is possible here. Their theories may be summarized very briefly by saying that the oestrogenic hormones of the ovary are thought to stimulate the pituitary to produce a specific hormone which acts directly on the mammary gland, causing growth of the duct system. This is followed by further stimulation of the pituitary by oestrin and progesterone with the result that another specific hormone is secreted which brings about the further arborization of the ducts and subsequent proliferation of the lobule-alveolar system. They also suggest that at least seven pituitary principles are required for the control of normal lactation. Its initiation and maintenance require the lactogenic hormone, the adenotropic hormone and the principle affecting carbohydrate metabolism, while the other factors which are probably more concerned with the actual constituents of the milk are the thyrotropic and the parathyrotropic hormones and the principles affecting protein and fat metabolism. At the same time the authors of the bulletin very rightly emphasize that although considerable evidence for this theoretical scheme already exists, much of it still awaits elucidation and proof.

Two years ago the application of hormones to research on the larger animals such as the cow and goat had only just begun, but since then the results obtained with smaller animals and the availability of purer hormone preparations has led to a greatly increased output in this particular direction. A memoir by Asdell *et al.*(44) gives an account of their experiments of which several were carried out on goats. It should be remembered, however, that this paper was prepared for publication at the beginning of 1936 with the consequence that one or two of the conclusions would now probably have to be revised. For example, in paragraph 12 of the summary the authors state that in causing mammary development the ovarian hormones act directly on the mammary gland or at any rate not through the hypophysis, a view

which in light of later work already cited has now been contradicted. One very interesting point in their conclusions is that prolactin injected into goats has no effect at the peak of lactation and only increases milk yield late in lactation and in the lowest yielding animals. Even then, the goats which respond ultimately show an unusually steep decline in lactation. The effect of prolactin on the yield and composition of the milk has also been studied by Folley & Young(45). They injected one cow daily for 15 days with 1 g. of their preparation in water at *p*H 8 and to another cow they gave water only. The hormone caused a 30% increase in daily milk yield while the fat content of the milk remained unaffected. The level of solids-not-fat on the other hand rose considerably and as this rise was accompanied by an increase in lactose and a decrease in chloride, the efficiency of the mammary gland was evidently greatly increased by the prolactin. During the period of richer milk the nitrogen partition was normal. It is of particular importance to observe that the effect was obtained with a prolactin preparation which was known to be practically free from either thyrotropic or gonadotropic activity. This particular point is by no means clear in what is otherwise a valuable contribution to the subject by Azimov *et al.*(46, 47) who have studied the effect of injecting *total* anterior pituitary preparations into 510 cows of various breeds and at different stages of lactation, using other 90 cows as controls. They showed that such injections resulted in a very marked though temporary rise in milk yield. In fact they claim that for all 510 animals there was the somewhat astounding net average gain of 15 l. of milk per head in two days. The highest yield was usually obtained 2 days after injection, when on one farm it was 121–129% above the mean recorded before injection and had only fallen to 105–111% a week later. Unfortunately the precise nature of the principle causing this phenomenal increase is not clear, for it appears that when purified prolactin preparations were used even in relatively large doses no effect was found, the great activity recorded occurring only with a *total* anterior pituitary extract. When this last fact is considered in conjunction with the work of Folley & Young(45) it is obvious that there is a very great necessity for further progress in the isolation from the anterior pituitary of lactogenic substances other than prolactin. That such work is in progress and will shortly be published by Folley & Young(48) is evident from the discussion appearing in a brief review of the subject by Folley(49), while matter of a similar nature is also receiving attention from Reece(50). Azimov *et al.* incidentally record that the effect of their treatment was greatest in animals which were well kept and well fed, and that it was absolutely harmless as regards both reproduction and milk secretion whether during the experiment or in subsequent lactations. Skarzinskaja(51), a colleague of Azimov, reports similar findings.

That hormone therapy has already begun to spread from the confines of the laboratory and to become of great practical importance is shown in such papers as that by Ross(52) who was able to increase the nursing ability of mothers by intramuscular injections of a prolactin preparation, and that by Kranenburg(53) who reports that therapy of a similar type had the same effect in mares and sows which were previously yielding insufficient milk for their young. In this connexion also it may be noted here that the opposite effect, the removal of unwanted lactation, has been effectively brought about in practice by oestradiol benzoate in women(54) and by oestrone in the bitch(55).

One of the greatest practical problems facing the dairy farmer to-day is why two cows bred according to the same principles of genetics and to all appearances so much alike should differ so markedly in their milk production, one giving a product far superior both in yield and composition to that of the other. Since the recent advances in endocrinology various workers have hoped to solve this problem by a study of hormones, and perhaps the best and most modern example has been published by

Reece & Turner(56, 57, 58) in which they describe their work on the prolactin and thyrotropic content of some 500 pituitaries from different bovine types such as steers, bulls, heifers and cows of different ages and at a variety of stages in their lactation and reproduction cycles. One of their objects was to find whether capacity to produce milk could in any way be related to the content of these two hormones in the hypophysis. It was found during the course of the work that pituitaries from heavily lactating cows were not readily available and so similar details for smaller animals were also collected as an aid towards interpreting the results for cows. The authors found that as the cow progresses from the foetal stage to complete maturity the average weight of the pituitary increases from 0·0431 g. in the foetus to 0·6485 g. in the calf, that it is 0·9025 g. when the age is between 4 and 10 months, 1·1590 g. between 11 and 23 months and 1·7521 at 2 years or over. As the female animal matures there is an increase in prolactin in the pituitary due both to the greater size of the gland itself and also to the rise in the prolactin content of the gland. In the male, on the other hand, the increase with age is due only to the enlargement of the gland and not to the lactogen level within it. The prolactin content of pituitaries from dairy cows has been compared with those from beef cows, and in the dairy cow the value has been found to be consistently higher. Thus when lactating and non-pregnant, and also when both lactating and pregnant, dairy cows have some 70% more lactogen than similar beef cows. With regard to the amount of thyrotropic hormone present in the pituitary, there was again an increase as the cow matured, and beef cows were found to have less than dairy cows at a corresponding stage. Although so numerous, the data presented in this bulletin(56) must only be regarded as a preliminary contribution to the scheme as a whole, but the authors seem confident that it will ultimately be possible for the milk-producing capacities of dairy cattle to be judged much more reliably than they can be to-day by new tests based on their endocrine systems.

Turning now to the gonadotropic hormones, the effect of gonadotropic preparations on the ovaries of the calf and cow has been investigated by Casida(59), who injected 1–3 months old animals with various doses of anterior pituitary extracts from sheep and also with pregnant mare serum. It was found that the pituitary extract did not cause ovulation as quickly as the mare serum but that it was much more potent in producing an increase in the weight of the ovaries. With mature cows the serum had little or no effect, while the anterior pituitary extract gave a fourfold increase in the weight of the ovaries. Casida(60) has also published a short review on the preparation, properties and use of gonad-stimulating hormones.

The oestrogenic hormones have also received much attention during the past two years. For example the influence of oestrogens on milk production in cows has been tested by different workers. Waterman *et al.*(61) treated the udder of a cow externally with oestradiol benzoate in olive oil with the result that the udder was reduced in size and the milk yield fell from 5 to 0·5 l. Another cow was treated in the same way with olive oil only, but without effect. After 33 days prolactin was injected, when the yield for the experimental animal increased to 50% of the original figure. Oestrogens have also been used in a series of experiments by Folley(62) of which part was cited in the previous review. He injected three cows intramuscularly with dihydrofollicular-hormone benzoate and to one of them he also gave oestrone. The results suggested that once a high level of oestrogens was attained in the blood, there was a decided inhibition of lactation in so far as the milk yield was reduced by some 20%. The milk itself, however, was richer both in fat and in solids-not-fat, and there was a very marked but temporary increase in milk phosphatase. With regard to the total daily output of solids, for one cow the hormone treatment appeared to increase the general efficiency of the gland notwithstanding the actual decrease in yield, but with the other two animals this increase in efficiency was not

so apparent. A study of the nitrogen partition showed that although the total nitrogen was increased in the milk the oestrogens did not appear to affect its distribution in any way, and consequently it must be concluded that these hormones do not tend to cause the production of colostrum. The calcium and phosphorus metabolism was also studied during the hormone treatment, when it was found that the injections caused a temporary fall in serum calcium followed by a very considerable but transient rise in serum phosphatase, observations which led Folley to assume that oestrogens may have a very considerable effect on the skeletal system.

In view of the previous divergence of opinion on the inhibitory action of oestrogens and of crystalline progesterone on lactation in gonadectomized rats, Folley & Kon (63, 64) have re-examined the question. They found that oestradiol monobenzoate administered to normal animals caused a considerable inhibition of lactation as measured by the growth rates of the young. But with rats ovariectomized on the day following parturition the inhibitory effect of similar doses of the same substance though definite was much less marked, while control animals ovariectomized after parturition and injected with sesame oil lactated satisfactorily. Progesterone on the other hand had no effect. Folley & Kon, who also studied the effect of the male hormones testosterone and androsterone, draw attention to the interesting fact that the ability of a hormone to cause development of the mammary gland appears to accompany the power to inhibit lactation. Thus oestrone, oestriol, oestradiol and testosterone are inhibitors of lactation but cause growth of the mammae, while progesterone and androsterone do neither. This action of testosterone, originally observed by Seyle *et al.* (65, 66, 67), has now been shown by them (68) to require the presence of the pituitary gland before mammary development is induced. In this it is again similar to the female sex hormones. Robson (69), working with mice, confirms the fact that testosterone inhibits lactation while androsterone does not, and finds also (70) that testosterone is much more effective than progesterone in the experimental inhibition of oestrus. Further researches on the effect of several substances of the male sex hormone group such as testosterone, androsterone, dehydroandrosterone and others on normal, spayed and hypophysectomized female rats have been carried out by Nelson & Merckel (71). Amongst the many observations made by these workers is that dehydroandrosterone produces periods of prolonged oestrus both in normal and in hypophysectomized animals. They suggest that this action may be due to the conversion of this substance to oestrogens by the ovary, a view which, if established, would indicate that ovarian function in this respect is not entirely suppressed by the removal of the pituitary. The combined action of male and female hormones in the rat has been studied by Hain (72), while Bottomley & Folley (73, 74) have investigated the effect of androgenic substances on the rate of teat growth and on the testes and accessory reproductive organs of the male guinea-pig. Further work which may be cited here has been published by Hisaw *et al.* (75) on progesterone; by Macdonald (76), Grant (77), Nelson (78) and Tejedor *et al.* (79) on different aspects of the reponses of the mammary gland to stimulation by the ovarian hormones; by Gillard (80) on the effects of hysterectomy on mammary development and by Hamilton & Gardner (81) on the effect of androgens on pregnant rats. This last treatment was found to cause pseudohermaphroditism in the female young.

With regard to the adrenal cortical hormone, Fitzhugh (82) has studied its effect on growth and sexual activities in rats. He made exact measurements of follicular and uterine diameters and found that the extract contained a female sex stimulating factor. It was observed that complete adrenalectomy suppressed the oestrous cycle while adequate administration of the extract restored it. On the chemical structure of corticosterone a note has been published by Steiger & Reichstein (83) confirming the theory previously put forward that this hormone possesses a steroid type of structure.

The effect of thyroxine on milk secretion in cows, a topic already discussed in the previous review, has now been investigated in still greater detail by Herman *et al.*(84) who find that the feeding of some 60 g. of desiccated thyroid gland daily to cows after the peak of lactation for a period of 8 weeks causes average increases in milk yield and butterfat of 18 and 35% respectively, whereas both at the height of lactation and towards the end the same treatment is almost without effect. Similar results were obtained by the injection of different amounts of thyroxine after the peak of lactation, but at the peak and towards the end this treatment appeared to result not in an increase but actually in a diminution of milk and fat production. From the practical point of view it is somewhat disappointing to learn that at present the amount of thyroxine required to produce these increases in milk and fat secretion is very much greater when given orally than when injected. It is probable that some inactivation of thyroxine takes place during rumination.

The importance of the thyroid gland in the lactation of small animals such as the rat and guinea-pig has been further investigated by Nelson & Tobin(85) and by Folley(86). In view of the previous work from different research schools showing the stimulating effect of thyroxine on milk secretion, it is surprising to find that Nelson & Tobin come to the conclusion that lactation in the rat is unaffected by thyroidectomy. Whatever may be the explanation of their results, it is now abundantly clear from Folley's experiments that removal of the thyroid and parathyroid tissue from rats during lactation causes a considerable decline in milk secretion, and that the same treatment before mating, though followed ultimately by normal parturition, results in very subnormal lactation. The importance of the thyroid gland in this respect is therefore again confirmed.

Many of the recent advances in endocrinology as applied to lactation have been due, firstly, to the preparation of pure hormones which can replace the crude extracts previously used, and secondly, to the more accurate methods of assay which have been introduced for their estimation. In these two respects progress is reported in the literature of the past two years. Lyons(87) has described a method for obtaining preparations of prolactin and of the adrenotropic hormone from sheep pituitaries, the glands being ground and extracted with acidified acetone. The extract so obtained is then treated with ammoniacal acetone and ultimately a protein is precipitated at *p*H 5·5 which has high prolactin activity. Another protein precipitated at *p*H 6·5 contains only a tenth of the prolactin of the other but is rich in the adrenotropic factor, which was tested for by Moon(88). In the paper by Lyons there is also a brief discussion on methods of assay with illustrations. Four methods for preparing a prolactin concentrate have been compared by Bergman & Turner(89) who considered that the process involving 60–70% alkaline alcohol extraction described by Bates & Riddle(90) is superior to those involving either aqueous alkali or acid acetone. A sample of prolactin as prepared by the method of Lyons was subjected to further purification by White *et al.*(91) who, by treatment with acetic acid and pyridine, prepared from it a crystalline protein. The crystals finally obtained after many treatments had a high prolactin activity, but this was scarcely high enough to justify the assumption that the crystalline substance actually was the hormone, particularly as in the authors' opinion the X-ray diffraction pattern and chemical analysis did not exclude the possibility that traces of some amorphous substance were still present. It is hoped that further research in the near future will lead to the preparation of the pure active principle itself, for the administration of prolactin would then be placed on a reliable chemical basis, and it would be possible definitely to differentiate it from other lactogens of the anterior pituitary. The chemistry of the purest preparations so far obtained have been investigated by McShan & French(92), who found them to be protein in nature, though again it does not follow that the hormone

itself is identical with this protein. The effect of such chemical treatments as diazotization, enzyme digestion, heating in dilute acids and alkalis and digestion with reagents known to react with specific groups, was studied. With the exception of acetic anhydride at 25°, most of the treatments caused inactivation. When heated with water at 100° for 1 hr. at *p*H 7·4 the activity was maintained, but at *p*H 9·7 or 3·2 it was destroyed.

The assay of hormones is another important branch of the subject, for it is only when accurate and reliable methods of assay have been adopted that the researches of one school can be adequately compared with those of another. The usual methods for prolactin estimation depend on some form of measurement of the growth caused in the crop gland of the pigeon. But as Folley(49) points out, this method may not necessarily be satisfactory for the assay of the actual lactogenic properties of a preparation since it is quite possible that the power to stimulate growth of the crop gland belongs to chemical substances entirely distinct from those possessing the power to stimulate and maintain milk secretion. Until, however, a new method based only on lactogenic principles is worked out, the growth of the pigeon crop gland will probably continue to play a fundamental part in this type of work. Dyer(93) has recently described a method in which the preparation is injected three times daily into the pectoral muscles of pigeons for 4 days. On the fifth day the birds are killed and the glands weighed. A more rapid method is described by Leblond & Allen(94) who first give the pigeon 0·5 mg. colchicine followed by an intradermal injection of the hormone preparation. After 10 hr. the bird is killed and the crop lining examined for cell proliferation. Apparently the colchicine causes the arrest of cell division in the metaphase stage, thus making it relatively easy to count the dividing cells. Methods have also been discussed by Lyons(87) and by Valle(95). The latter suggests the use of pigeons provided with a permanent fistula of the crop so that mucosa cells may be scraped from the crop lining and examined, but his method does not appear to have any advantage over others in which the birds are killed. With regard to the estimation of prolactin, it is of interest to observe that just as this hormone stimulates lactation while oestrogens inhibit it, so it has been found by Folley & White(96) that the stimulating action of prolactin on crop growth in the pigeon is considerably inhibited by the simultaneous injection of oestrogens. Dealing with another hormone Van Dyke(97) suggests a possible method for the assay of progesterone by determining the doses required to cause contraction of the uterus in the non-pregnant cat in response to sympathetic stimulation, while Kober(98) has continued his researches on the colorimetric estimation of oestrone, and discusses its application to the assay of oestrogens in urine. Methods for the comparative assay of "mammogen", using the male mouse for the test, have been devised by Lewis & Turner(99).

If the exact nature of hormone action is ever to be thoroughly understood it is essential that as much information as possible should accumulate on the question of small changes in chemical constitution and their effect on hormonal activity and also on the existence of other chemical compounds possessing physiological activity similar to that attributed to the hormones themselves. An extensive contribution in this direction has been made by Miescher *et al.*(100, 101, 102) who have studied the biological activity of numerous esters of oestrone and oestradiol. Direct reference to the papers is essential as the results are much too numerous for adequate treatment in the present review. Robson(103) has found the action of triphenyl ethylene to be similar to that of the oestrogens, while Bottomley & Folley(104) found definite growth of the nipples in male guinea-pigs on the administration of *p*-anol or its benzoate, but in this case the activity though appreciable did not appear to be as great as with the oestrogens themselves. Extensive work on certain derivatives of the stilboestrols

and their biological activity has been carried out by Dodds *et al.*(105, 106) while Folley & Watson(107) have tested the effect of diethylstilboestrol on the composition of cow's milk. In this and in other biological tests its action closely resembles that of the oestrogens, and it has the advantage of being relatively easy to obtain.

Urine as a source of different hormones has again been discussed during the past two years. The preparation of an active lactogen from the urine of pregnant women has been described by Laugecker & Schenk(108) and from that of lactating women by Liard(109). The inhibition of the oestrus cycle with post-partum urine and with crude prolactin has been studied by Nathanson *et al.*(110) and the inhibition of lactation by an anterior-pituitary like preparation from pregnancy urine is reported by Connon(111). Urine of pregnancy or lactation appears to contain such a number of different hormones that it is little wonder that some of the observations at the present stage are difficult to interpret and often conflicting.

Biochemical aspects

The main principle underlying one of the most fruitful fields of research during the past few years has been the detailed analysis of cows' blood before and after its passage through the udder. This technique of simultaneous arterial and venous blood sampling has again been used by Maynard *et al.*(112), who tested different methods for obtaining arterial samples and decided to obtain them from the internal pudic artery to which they gained access through the vagina. Their results confirm those of other workers which were fully discussed in the previous review and which showed that the lipoid fraction of the blood used by the mammary gland as a precursor of milk fat is the neutral glyceride fraction and not the phosphatides or cholesteryl esters.

Further work based on the same principle has been carried out by Graham(113) who has developed a more elaborate technique than has hitherto been used. He and his colleagues obtained the arterial sample from the carotid artery which was first exteriorized by an operation described in a special bulletin(114). In one series of experiments(115) to investigate the balance of carbohydrate metabolism in the gland not only was this artery exteriorized but also one mammary gland was removed. Then, by tying and cutting the external pudic and perineal veins, only one major inlet and outlet was left for the blood passing through the remaining gland. The rate of blood flow was also measured. The results showed that in a given time much more lactose was secreted (e.g. 12·62 and 6·06 g.) than could be accounted for by the glucose taken up from the blood (5·15 and 3·16 g. respectively), and that some other precursor must play an essential part in the production of lactose. Further experiments showed that this precursor was most probably lactic acid, for relatively large amounts of this substance were found to be removed from the arterial blood, and when the carbohydrate balance was calculated, it was shown from two experiments that the probable amount of lactose arising from blood glucose and blood lactic acid respectively was some 53 and 33% of the total secreted, the remaining 14% possibly coming from amino acids. Further experiments by Shaw *et al.*(116), also using simultaneous arterial and venous sampling, have confirmed the fact that lactic acid is an important precursor of milk constituents. These authors conclude that all the lactose of the milk may be supplied by glucose and lactic acid, but obviously more work is required before this statement is finally proved. The same workers find that the amount of sugar removed from the arterial blood does not appear to be related to the period of time after milking or to the arterial blood sugar level or to the level of milk production. The last two points appear contradictory to the findings of Graham *et al.*(117) which were discussed in the previous review. The question as to whether the lactose content of the milk is related to blood sugar level

has been further investigated by Petersen & Boyd(118) who perfused the external pudic artery for 6 hr. with isotonic glucose solution. Although the mammary venous blood contained as much as 206–361 mg. glucose/100 ml. and sugar was being lost in the urine, there was no consequent increase in the lactose content of the milk.

With regard to the fact that lactose may arise from lactic acid and glucose, Petersen & Shaw(119) have examined the possibility of such a synthesis *in vitro* by adding macerated mammary tissue to solutions of glucose containing either lactic acid or its salts and incubating the mixture at 37° under toluene. Control experiments with mammary tissue alone showed nothing but the slightest trace of lactose, whereas in the others there were obtained relatively large amounts of a disaccharide which was proved by its osozone to be lactose. Unfortunately the present note on this subject by Petersen & Shaw is not sufficiently detailed to make it possible to judge whether or not the lactic acid was really an essential reactant. That it may not have been so seems possible from the work of Grant(120) who was able to synthesize lactose simply by incubating mammary tissue slices with glucose alone in physiological saline solutions, and from these mixtures more than negligible traces of lactic acid would presumably be absent. Nor does the work of Weinbach(121) make this point any clearer, for he substituted pig's serum for the saline solution as used by Grant and this would be expected to contain lactic acid in small amounts. When a more detailed account of their work is published by Petersen & Shaw, it may be possible to say whether or not the lactic acid really plays an essential part in the synthesis.

Turning again to the technique of arterial-venous blood sampling, Graham *et al.*(122, 123) have demonstrated by its means that urea is formed in small amounts in the actively lactating gland and that the chief nitrogenous constituents of the blood removed by the gland are globulin, amino nitrogen and unknown fractions of the non-protein nitrogen, globulin appearing to be the chief source of this element. It is of particular interest to observe that for 1 or 2 hr. immediately after milking the gland appeared to be in a state of negative equilibrium from which it gradually changed to a positive balance, a condition in which it remained until the next milking. This led to the suggestion that nitrogen secreted in the milk immediately after milking may largely be derived from nitrogenous compounds which have been stored in the gland towards the end of the previous interval. That urea may be formed in the lactating gland is confirmed by Shaw & Petersen(124) who find that appreciable quantities of arginase exist in the active tissue but that very little if any exists in the non-lactating gland. These authors(125) have also confirmed previous work by showing that the amount of amino-acid nitrogen absorbed from the blood by the active gland cannot account for more than 35% of the milk proteins. At the same time they suggest that the uric acid, creatine and creatinine of the blood are not important precursors of milk constituents. It is therefore again made clear that the blood proteins must play a very active part in the synthesis of caseinogen.

Arterial and venous sampling has also been used in a further set of experiments by Graham *et al.*(126) to calculate the efficiency of the mammary gland of the goat from data obtained by measuring the oxygen uptake and carbon dioxide output as the blood passes through the gland. Only for 3 out of 26 determinations was the respiratory quotient less than unity, the maximum value being 1·92 and the mean 1·36. This would suggest that carbohydrate material is used by the gland for fat synthesis as well as for the energy of metabolism. By making this deduction and by knowing the normal ratio of blood flow to milk yield and the energy of the milk produced, Graham and his colleagues were able to calculate that over 90% of the energy received by the gland from the blood is contained in the milk secreted and that less than 10% is necessary for the actual transformation of blood precursors to milk constituents. It may therefore be assumed, provided the part played by the

lymph is very small, that the gross synthetic efficiency of the mammary gland reaches a high figure of over 90%.

In connexion with the possible synthesis of fat in the gland as suggested by Graham, it is evident that work is shortly to be published by Shaw & Petersen(127) which does not agree with this hypothesis, but rather suggests that sufficient fat is removed from the blood to account for the milk fat and that some of it may even be oxidized in the gland to supply energy. It will be interesting to find from the detailed description of the work how these opposite views may be reconciled.

Further work on the secretion of milk fat continues to be published from various research schools. Bulletins by McCandlish(128) and by Turner(129) summarize very clearly many of the views at present held with regard to the effects on milk fat production of various important factors including, amongst others, breed, individuality, age and stage of lactation, and oestrus.

A study of milk fat secretion problems by means of "labelled" fats has been made by Aylward *et al.*(130) and by McConnell & Sinclair(131). The former fed fat partially saturated with iodine to cows and followed the course of the iodized fat in the blood and milk. It was found that although the blood fat level in the ruminant does not measurably increase after a fatty meal, yet it does become enriched by the constituents of the dietary fat. There was also found to be a direct relationship between the amount of the labelled fat in the milk and the amount in the blood, particularly in the non-phosphatide fraction of the blood, the maximum amount in the milk being secreted two days after the fat was administered. McConnell & Sinclair fed to pregnant rats diets rich in fat containing elaidic acid, a substance which can be detected and estimated by the fact that it is both solid and unsaturated, two properties which do not normally occur together in fatty acids of animal origin. They found that the liver phosphatide of the new-born young contained 16% of elaidic acid and that the fatty acid of the entire bodies contained 11%, a figure which rose to 61% after 10 days' suckling. Dietary fat in the rat can therefore pass through the placenta and also into the milk of the mother. It is clear therefore that at least a portion of the fat secreted by the mammary gland probably arises directly in normal circumstances from the fat of the diet. Further work on the influence of dietary fat on certain properties of butter such as its degree of unsaturation and its hardness has been described by Hill & Palmer(132).

The effect of cod-liver oil fed to cows in changing the nature and amount of the milk fat is now well known. The factor responsible for this change has been traced to the saponifiable portion of the oil, and is thought to be due to higher unsaturated acids, a suggestion which receives support from the work of McCay *et al.*(133) who have shown conclusively that partial hydrogenation of the oil prevents its adverse action on milk fat secretion. That salmon oil did not appear to have the same consistent effect on the milk fat may be due to the fact that this oil is not obtained solely from the liver of the fish but from the body as a whole. It would therefore seem that the harmful constituent may be some kind of unsaturated acid occurring mainly in the liver of the fish, but further work is still required before this peculiar effect is thoroughly understood. Graham & Cupps(134) have also studied the subject, but owing to the fact that they used herring oil, which did not appear to have much effect, and that they encountered difficulties in connexion with food intake while it was being administered, their results are not conclusive.

If the physiological processes involved in the secretion of milk fat are ever to be thoroughly understood it is essential that the precise nature of milk fat should be known. The results of Hilditch *et al.*(135, 136, 137) confirm previous work in proving the existence in butter fat of lower unsaturated acids such as decenoic, dodecenoic and tetradecenoic. They have further shown conclusively that the double bond in all

the lower unsaturated acids occupies the same position in the molecule as it does in oleic acid and again put this forward as evidence for the possible formation in the mammary gland of glycerides containing lower fatty acids by the oxidation and reduction of glycerides containing oleic acid. A theory involving fat oxidation is evidently also supported by Shaw & Petersen(127) but at the same time it must be noted that if fats are synthesized at all in the mammary gland from carbohydrates or other substances, as is suggested by the work of Graham *et al.*(126), then it would appear at least equally probable that these lower unsaturated acids may be intermediates of synthesis rather than of breakdown, particularly as diets rich in fat are known to cause a decrease, and diets rich in carbohydrate an increase, in the normal content of the lower fatty acids in milk fat. At the present time further evidence is still required before the existence of these lower acids in milk can be explained.

The possible site of fat synthesis in the mammary gland forms the subject of a paper by Kelly & Petersen(138). Sections of the gland fixed in 10% formalin and physiological saline, were stained with five different dyes differentiating neutral fat and free fatty acids. Free fatty acids were found to be present in greatest amount in the basal part of the epithelial cell decreasing towards the lumen and appearing to be non-existent in the alveolus. These free fatty acids had a Reichert Meissl value twice that of normal milk fat, which suggested that they were actively concerned in its production. As part of a scheme for a general study of the biochemistry of milk fat secretion, the lipoid level and the distribution of the lipoids of whole blood during inanition and realimentation has been studied in detail in the first instance for the non-lactating ruminant by Aylward & Blackwood(139). Only in the phosphatide fraction did they find any consistent alteration in level even during the rigours of a 10½-day fast, the total fatty acid content showing no significant change.

Other work which may be cited on problems connected with milk fat has been published by Rewald(140) and Holm *et al.*(141) on milk phosphatides, by Chollet & Camus(142) on the various constants for the milk fat from both cows and goats, and by Stebnitz & Sommer(143) on the stability of butter fat towards oxidation. The latter authors show that the stability is inversely related to the unsaturation of the fat, which, in turn, depends partly on the diet and partly on the individuality of the animal.

A new technique involving the use of radioactive phosphorus has been applied with most marked success to milk secretion problems by Aten & Hevesy(144). Sodium phosphate containing radio-active phosphorus atoms was injected into goats and its entry into various constituents of the blood and milk was studied. Among the various far-reaching observations made by the authors are these: (1) The inorganic phosphorus of the milk showed considerable radio-activity even 2 hr. after injection, and it took 3–4 hr. for it to be composed almost entirely of individual atoms which had been present in the plasma after the beginning of the experiment. (2) Caseinogen phosphorus appeared to come from the inorganic phosphorus of the plasma and the actual formation of the protein by the gland appeared to take about an hour. (3) Ester phosphorus also arose from inorganic phosphorus, but when radio-active ester phosphorus was administered, the inorganic phosphorus of the milk was found to contain the radioactive element. There was therefore rapid breakdown and synthesis of ester phosphorus in the gland. (4) Plasma phosphatides were shown not to give rise to milk fat and inorganic phosphorus in the gland, thus confirming previous work by others which has shown that blood phosphatides are not amongst the precursors of milk.

One line of investigation which may possibly lead to valuable information on the physiology of lactation is a study of milk and lactation in carnivorous animals as compared with herbivorous. It is interesting to note therefore that an apparatus for

milking small laboratory animals such as rats has been introduced by Cox & Mueller(145) and modified by Temple & Kon(146). The former authors with the aid of this apparatus have been able to compare the composition of rats' milk with that of humans and cows, while Houston *et al.*(147) have used it for studying the vitamin content of milk from rats and guinea pigs. The value of an efficient apparatus of this type cannot be overestimated since so much research work on milk secretion has necessarily to be carried out on the small laboratory animal.

Biophysical aspects

Further research on the anatomy of the mammary gland has been published by Becker(148) who has examined in particular the flow of venous blood from the udder. In spite of the view generally held that the udder possesses three pairs of efferent veins, the subcutaneous abdominal, the external pudic and the internal pudic, he finds that the direction of blood flow in the last of these is actually into the udder rather than away from it and that the internal pudic vein can therefore no longer be regarded as efferent. An exceedingly interesting paper dealing with the comparative conformation and anatomy, and udder characteristics of cows of both beef and dairy breeds has been published by Swett *et al.*(149). The work is most efficiently described and excellently illustrated, but is too detailed for a brief review such as is permissible here. The discussion and summary is clear and concise and is well worth perusal. Further data collected in the study of the anatomy of nearly 600 dairy cows for which records of production are available have also been published in tabulated form by Swett and his colleagues(150), while in a further paper(151) they draw attention to the difficulty of obtaining or computing accurate production records for the comparative study of milk capacity when one or more quarters are non-lactating. They show the error of endeavouring to compensate for the loss of a quarter by calculating what the quarter would have been expected to yield had it been functioning normally, since in the anatomy of the udder itself in such circumstances there is found to be a strong tendency towards a natural compensation in the greatly enhanced producing capacity of the remaining quarters, particularly if the loss suffered occurs when the animal is young. The secretion of milk fat from different quarters of the udder is discussed by Skrodel(152) who observed that in 73 cows the right and left halves of the udders gave similar yields, while in 85% of the animals the forequarters gave less than the hind, though this difference lessened as the number of lactations increased. He also suggests(153) that the first quarters to be milked tend to give more milk and milk fat than the others, and that a procedure of milking in which the poorer yielding quarters are milked first results in a greater yield. He finds that a greater yield is also fostered by a milking process which is rapid rather than slow. Hooft(154), in a study of the mammary gland, finds that during its development epithelial and connective tissue elements are formed at similar rates and that adipose tissue is changed to a cellular and reticular tissue preceding the period of glandular activity. In these observations he has apparently confirmed the earlier work of Florentin(155).

In studying the subject of milking capacity of cows and various factors which affect it, Kronacher & Hogreve(156) have endeavoured to find whether there is any correlation between blood grouping and milking capacity. Some 111 animals contained in three different herds were examined. The most common group Oo was found in 51, 37 and 82% of the three herds respectively. The next most common group was Ao, and several others also occurred to a lesser extent. No definite correlation could be found between blood grouping and milk yields during the second, third and fourth lactations except that animals in the Oo group had yields falling near the herd average. For the other groups the results were contradictory. Farkas(157) by

collecting data for 627 cows has shown that there is a positive correlation ($0{\cdot}83 \pm 0{\cdot}01$) between the highest daily milk yield and the yield for the whole lactation. In fact for every litre increase in yield per day he finds an increase of 185 l. in total milk production during the lactation, showing that the highest daily yielder tends also to be the best producer in the whole lactation.

In the previous review attention was drawn to the work carried out on the involution of the mammary gland and particularly to the conclusion of Selye(158) that involution of the mammary gland after weaning was conditioned by the withdrawal of the nervous stimulation of suckling rather than by accumulation of the products of secretion. In view of this work Reece & Turner(159, 160, 161) were led to study the influence of suckling upon the prolactin content of the rat pituitary and have shown that when suckling is stopped the accumulation of milk in the gland is accompanied by a threefold rise of prolactin in the pituitary gland. By ligating the primary duct of each mammary gland in one group of rats so that no milk could be withdrawn it was found that the act of suckling alone even without the removal of milk is sufficient to reduce the prolactin content of the pituitary from an average of 9·2 to one of 5·2 units, a corresponding figure of 3·1 being obtained when the young were allowed to suckle and remove milk freely. It would therefore appear that it is the cessation of suckling rather than the accumulation of milk in the gland which affects the prolactin content of the pituitary as well as the involution of the mammary gland itself. Experiments on the effect of X-rays or of ultraviolet light on the function of the mammary gland are reported by Bognewskii(162), Becchini & Carteni(163) and Antonov(164).

Clinical chemistry of dairy cattle

Ever since their importance in the maintenance of good health in dairy cows was realized, the level of calcium, phosphorus and magnesium in the blood under different pathological and physiological conditions has continued to receive considerable attention. Van-Landingham *et al.*(165) have continued their previous researches with particular reference to the calcium and inorganic phosphorus of the whole blood of Holstein heifers during their first gestation and lactation. They find that both parturition and lactation cause a marked drop in the phosphorus content of the blood, particularly on a diet low in phosphorus, but that this decrease can be avoided to a large extent by normal supplies of that element in the food. They also note that the lower levels of phosphorus do not appear to have any appreciable effect on the calcium content of the blood. The same authors(166) have estimated the inorganic phosphorus level in over 200 samples of whole blood from twenty-two Holstein dairy cows at 8-week intervals. They found a decline in the level from the first, through the second, to the third lactation, the average figures in the three periods being respectively 4·95, 4·81 and 4·35 mg. phosphorus/100 ml. blood, and there appeared to be no further fall in subsequent lactations. The inorganic phosphorus of the serum for a large number of samples from Holstein and Jersey cattle of all ages are recorded by Eveleth *et al.*(167). There is evidence of a slight breed difference, the level for the Jerseys being lower than that of the Holsteins during the first 2 years.

The calcium level in 160 samples of normal plasma from cattle has been estimated by Haig(168). He finds the values to range between 8·05 and 11·48 mg./100 ml. with an average of 9·99, more than 90% of the figures falling between 9·0 and 11·0 mg. Again, phosphate phosphorus was found to be considerably affected by various factors, including age and phosphorus intake, but the normal figure for mature cattle appeared to be about 5 mg./100 ml. Work by Aylward & Blackwood(139) on the effect of fasting and realimentation on the calcium and phosphorus metabolism of non-lactating cows shows that during inanition there is a continuous and effective mobil-

ization of these two elements from skeletal tissue with the result that their level in the blood is approximately maintained throughout the fast even although there is continual loss of these elements in the urine and faeces.

The magnesium content of blood plasma has been determined by Duncan & Huffman(169) for 107 calves over a period of 3 years at intervals of 1–2 weeks. For over two thousand values the mean figure was found to be 2·41 mg./100 ml. plasma, the range being 1·62–3·83 mg., while 80% of the figures fell between 2·0 and 2·8 mg. Seasonal changes in the individual figures are discussed and the authors make the important statement that the range of normal variation is sufficiently great to include many of the variations which occur under pathological conditions. In the light of this observation it is interesting to note that the importance of magnesium level is stressed by Moore *et al.*(170) who describes certain pathological conditions in calves which they associate with a previous history of low magnesium content.

The fixation of calcium, inorganic phosphorus and magnesium to the proteins of serum has received attention from Seekles(171). He finds that the proportion of the total calcium which is diffusible is only slightly decreased by a change in the blood towards the alkaline side but that it is markedly increased by a shift in a more acid direction until at *p*H 4·1 all the calcium becomes diffusible. The author raises the question as to whether a shift from the normal *p*H of 7·4 to 7·2 or 7·1 would increase the ionized calcium sufficiently to alleviate paresis in milk fever.

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REFERENCES

(1) CRAIG (1936). *Vet. Rec.* **48**, 1503.
(2) LAGERLÖF (1935). *Z. Zücht. B*, **32**, 47.
(3) PHILLIPS & ANDREWS (1936). *Bull. Mass. agric. Exp. Sta.* no. 331.
(4) ARNOLD, NEAL & THOMAS (1933–5). *Proc. Ass. Sth agric. Wkrs*, 525.
(5) MEIGS & CONVERSE (1936). *J. Dairy Sci.* **19**, 438.
(6) —— —— (1938). *J. Dairy Sci.* **21**, 114.
(7) SAMPSON, BOLEY & GRAHAM (1938). *Cornell Vet.* **28**, 52.
(8) PEARSON (1937). *Amer. J. Physiol.* **118**, 786.
(9) PEARSON, HART & BOHSTEDT (1937). *J. Nutrit.* **14**, 329.
(10) ANDERSON (1936). *Emp. J. exp. Agric.* **4**, 186.
(11) ANDERSON (1936). *Emp. J. exp. Agric.* **4**, 197.
(12) CHAPMAN & CASIDA (1937). *J. agric. Res.* **54**, 417.
(13) QUINLAN & ROUX (1936). *Onderstepoort J. vet. Sci.* **6**, 719.
(14) REECE & TURNER (1938). *J. Dairy Sci.* **21**, 37.
(15) ANDERSON (1936). *Vet. J.* **92**, 200.
(16) MILLER (1936). *Vet. Rec.* **48**, 903.
(17) SATO (1937). *J. Chosen Med. Ass.* **27**, 280.
(18) ROOS & ROMIJN (1938). *J. Physiol.* **92**, 249.
(19) DICKERSON (1937). *J. Dairy Sci.* **20**, 437.
(20) LABES (1937). *Dtsch. landw. Tierz.* **41**, 540.
(21) TURNER (1937). *J. Dairy Sci.* **20**, 436.
(22) NELSON (1935). *Proc. Soc. exp. Biol., N.Y.*, **33**, 222.
(23) ASDELL & SEIDENSTEIN (1935). *Proc. Soc. exp. Biol., N.Y.*, **32**, 931.
(24) SELYE, COLLIP & THOMSON (1935). *Proc. Soc. exp. Biol., N.Y.*, **32**, 1377.
(25) SELYE, BROWNE & COLLIP (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 198.

(26) Selye, Browne & Collip (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 472.
(27) Reece, Turner & Hill (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 204.
(28) Lyons & Pencharz (1936). *Proc. Soc. exp. Biol., N.Y.*, **33**, 589.
(29) Gomez & Turner (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 320.
(30) —— —— (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 404.
(31) —— —— (1936). *Proc. Soc. exp. Biol., N.Y.*, **35**, 59.
(32) —— —— (1936). *Proc. Soc. exp. Biol., N.Y.*, **35**, 365.
(33) —— —— (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 78.
(34) —— —— (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 80.
(35) —— —— (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 286.
(36) —— —— (1936). *J. Dairy Sci.* **19**, 450.
(37) —— —— (1937). *J. Dairy Sci.* **20**, 410.
(38) —— —— (1938). *Proc. Soc. exp. Biol., N.Y.*, **37**, 607.
(39) Gomez, Turner, Gardner & Hill (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 287.
(40) Gomez & Turner (1937). *Res. Bull. Mo. agric. Exp. Sta.* no. 259.
(41) Nelson & Gaunt (1937). *Pro. Soc. exp. Biol., N.Y.*, **36**, 136.
(42) Hall, Chamberlin & Müller (1938). *Amer. J. Physiol.* **122**, 16.
(43) Pencharz & Lyons (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 388.
(44) Asdell, Brooks, Salisbury & Seidenstein (1936). *Mem. Cornell agric. Exp. Sta.* no. 198.
(45) Folley & Young (1937). *J. Soc. chem. Ind., Lond.*, **56**, 96.
(46) Azimov, Krouze, Skarzinskaja, Mahova & Fominskaja (1936). *Probl. Zivotn*, **8**, 28. *Animal Breed. Abstr.* **5**, 21.
(47) Azimov & Krouze (1937). *J. Dairy Sci.* **20**, 289.
(48) Folley & Young (1938). *Proc. roy. Soc.* B, **126**, 45.
(49) Folley (1938). *Lancet*, **2**, 389.
(50) Reece (1938). *J. Dairy Sci.* **21**, 117.
(51) Skarzinskaja (1936). *Usp. zooteh. Nauk*, **3**, 67. *Anim. Breed. Abstr.* **5**, 401.
(52) Ross (1938). *Endocrinology*, **22**, 429.
(53) Kranenburg (1936). *Tijdschr. Diergeneesk.* **63**, 185. *Vet. Bull.* **7**, 37.
(54) Ramos & Colombo (1938). *Dtsch. med. Wschr.* **782**. *Lancet*, **2**, 30.
(55) Witzigmann (1938). *Tierärztl. Rdsch.* **1**, 7. *Vet. Rec.* **50**, 492.
(56) Reece & Turner (1937). *Res. Bull. Mo. agric. Exp. Sta.* no. 266.
(57) —— —— (1936). *J. Dairy Sci.* **19**, 448.
(58) —— —— (1936). *Proc. Soc. exp. Biol., N.Y.*, **35**, 60.
(59) Casida (1936). *J. Dairy Sci.* **19**, 449.
(60) —— (1938). *J. Dairy Sci.* **21**, 101.
(61) Waterman, Freud & de Jongh (1936). *Acta brev. neerl. Physiol.* **6**, 84. *Anim. Breed. Abstr.* **5**, 18.
(62) Folley (1936). *Biochem. J.* **30**, 2262.
(63) Folley & Kon (1938). *Proc. roy. Soc.* B, **124**, 476.
(64) —— —— (1937). *Nature, Lond.*, **139**, 1107.
(65) Selye, McEuen & Collip (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 201.
(66) —— —— —— (1936). *Amer. J. Physiol.* **116**, 29.
(67) —— —— —— (1936). *Proc. Soc. exp. Biol., N.Y.*, **35**, 56.
(68) McEuen, Selye & Collip (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 2, 213.
(69) Robson (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 153.
(70) —— (1937). *J. Physiol.* **90**, 15*P*.
(71) Nelson & Merckel (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 823.
(72) Hain (1937). *Quart. J. exp. Physiol.* **26**, 293.
(73) Bottomley & Folley (1938). *J. Physiol.* **92**, 15*P*.
(74) —— —— (1938). *J. Physiol.* **92**, 33*P*.

(75) Hisaw, Greep & Fevold (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 840.
(76) Macdonald (1936). *Surg. Gynec. Obstet.* **63**, 138.
(77) Grant (1937). *Biochem. J.* **31**, 1538.
(78) Nelson (1937). *Amer. J. Anat.* **60**, 341.
(79) Tejedor, Teresa & Contento (1936). *Milchw. Forsch.* **18**, 79.
(80) Gillard (1937). *Amer. J. Physiol.* **120**, 300.
(81) Hamilton & Gardner (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 570.
(82) Fitzhugh (1937). *Amer. J. Physiol.* **118**, 677.
(83) Steiger & Reichstein (1938). *Nature, Lond.*, **141**, 202.
(84) Herman, Graham & Turner (1938). *Res. Bull. Mo. agric. Exp. Sta.* no. 275.
(85) Nelson & Tobin (1937). *Endocrinology*, **21**, 670.
(86) Folley (1938). *J. Physiol.* **93**, 401.
(87) Lyons (1937). *Proc. Soc. exp. Biol., N.Y.*, **35**, 645.
(88) Moon (1937). *Proc. Soc. exp. Biol., N.Y.*, **35**, 649.
(89) Bergman & Turner (1937). *J. Biol. Chem.* **118**, 247.
(90) Bates & Riddle (1935). *J. Pharmacol.* **55**, 365.
(91) White, Catchpole & Long (1937). *Science*, **86**, 82.
(92) McShan & French (1937). *J. biol. Chem.* **117**, 111.
(93) Dyer (1936). *J. Physiol.* **88**, 6 *P.*
(94) Leblond & Allen (1937). *C.R. Soc. Biol., Paris*, **124**, 1190.
(95) Valle (1937). *C.R. Soc. Biol., Paris*, **126**, 134.
(96) Folley & White (1937). *Nature, Lond.*, **140**, 505.
(97) Van Dyke (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 11.
(98) Kober (1938). *Biochem. J.* **32**, 357.
(99) Lewis & Turner (1938). *J. Dairy Sci.* **21**, 119.
(100) Miescher, Scholz & Tschopp (1938). *Biochem. J.* **32**, 141.
(101) —— —— —— (1938). *Biochem. J.* **32**, 725.
(102) —— —— —— (1938). *Biochem. J.* **32**, 1273.
(103) Robson (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 153.
(104) Bottomley & Folley (1937). *J. Soc. chem. Ind., Lond.*, **56**, 1054.
(105) Dodds, Goldberg, Lawson & Robinson (1938). *Nature, Lond.*, **141**, 247.
(106) —— —— —— —— (1938). *Nature, Lond.*, **142**, 34.
(107) Folley & Watson (1938). *Lancet*, **235**, 423.
(108) Laugecker & Schenk (1936). *Med. Klin.* **33**, 1104.
(109) Liard (1937). *C.R. Soc. Biol., Paris*, **126**, 512.
(110) Nathanson, Fevold & Jennison (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 481.
(111) Connon (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 52.
(112) Maynard, McCay, Ellis, Hodson & Davis (1938). *Mem. Cornell. agric. Exp. Sta.* no. 211.
(113) Graham (1937). *J. Dairy Sci.* **20**, 413.
(114) Graham, Turner & Gomez (1937). *Res. Bull. Mo. agric. Exp. Sta.* no. 260.
(115) Graham (1937). *J. biol. Chem.* **122**, 1.
(116) Shaw, Boyd & Petersen (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 579.
(117) Graham, Jones & Kay (1936). *Proc. roy. Soc.* B, **120**, 330.
(118) Petersen & Boyd (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 537.
(119) Petersen & Shaw (1937). *Science*, **86**, 398.
(120) Grant (1935). *Biochem. J.* **29**, 1905.
(121) Weinbach (1936). *J. gen. Physiol.* **19**, 829.
(122) Graham, Houchin & Turner (1937). *J. biol. Chem.* **120**, 29.
(123) Graham, Petersen, Houchin & Turner (1938). *J. biol. Chem.* **122**, 275.
(124) Shaw & Petersen (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 631.
(125) —— —— (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 632.

(126) Graham, Houchin, Peterson & Turner (1938). *Amer. J. Physiol.* **122**, 150.
(127) Shaw & Petersen (1938). *J. Dairy Sci.* **21**, 122.
(128) McCandlish (1936). *Bull. West Scot. agric. Coll.* no. 132.
(129) Turner (1936). *Res. Bull. Mo. agric. Exp. Sta.* no. 365.
(130) Aylward, Blackwood & Smith (1937). *Biochem. J.* **31**, 130.
(131) McConnell & Sinclair (1937). *J. biol. Chem.* **118**, 123.
(132) Hill & Palmer (1938). *J. Dairy Sci.* **21**, 529.
(133) McCay, Paul & Maynard (1938). *J. Nutrit.* **15**, 367.
(134) Graham & Cupps (1938). *J. Dairy Sci.* **21**, 45.
(135) Hilditch & Paul (1936). *Biochem. J.* **30**, 1905.
(136) Hilditch (1937). *Analyst*, **62**, 250.
(137) Hilditch & Longenecker (1938). *J. biol. Chem.* **122**, 497.
(138) Kelly & Petersen (1936). *J. Dairy Sci.* **19**, 448.
(139) Aylward & Blackwood (1936). *Biochem. J.* **30**, 1819.
(140) Rewald (1937). *Lait*, **17**, 225.
(141) Holm, Wright & Deysher (1936). *J. Dairy Sci.* **19**, 631.
(142) Chollet & Camus (1937). *Ann. Falsif. Paris*, **30**, 405.
(143) Stebnitz & Sommer (1937). *J. Dairy Sci.* **20**, 265.
(144) Aten & Hevesy (1938). *Nature, Lond.*, **142**, 111.
(145) Cox & Mueller (1937). *J. Nutrit.* **13**, 249.
(146) Temple & Kon (1937). *Biochem. J.* **31**, 2197.
(147) Houston, Kon & Henry (1938). *J. Soc. chem. Ind., Lond.*, **57**, 276.
(148) Becker (1937). *J. Dairy Sci.* **20**, 409.
(149) Swett, Miller, Graves, Black & Creech (1937). *J. agric. Res.* **55**, 239.
(150) Swett, Matthews, Miller & Graves (1938). *J. Dairy Sci.* **21**, 7.
(151) —— —— —— —— (1937). *Bur. Dairy Ind., U.S.* Dep. Agric., B.D.I.M. 589.
(152) Skrodel (1936). *Milchw. Forsch.* **18**, 27.
(153) —— (1937). *Milchw. Forsch.* **19**, 72.
(154) Hooft (1936). *Bull. Histol. Tech. Micr.* **13**, 178.
(155) Florentin (1936). *Bull. Histol. Tech. Micr.* **13**, 336.
(156) Kronacher & Hogreve (1936). *Z. Zücht. B*, 35, 89. *Milchw. Forsch.* **18**, Ref. 143.
(157) Farkas (1936). *Mezögazdas kutatas*, **9**, 254. *Nutr. Abstr. Rev.* **7**, 225.
(158) Selye (1934). *Amer. J. Physiol.* **107**, 535.
(159) Reece & Turner (1936). *Proc. Soc. exp. Biol., N.Y.*, **35**, 367.
(160) —— —— (1937). *Proc. Soc. exp. Biol., N.Y.*, **35**, 621.
(161) —— —— (1937) *J. Dairy Sci.* **20**, 411.
(162) Bognewskii (1936). *Milchw. Forsch.* **18**, Ref. 80.
(163) Becchini & Carteni (1936). *Milchw. Forsch.* **18**, Ref. 149.
(164) Antonov (1936). *Arch. Med. Enf.* **39**, 216.
(165) Van Landingham, Henderson & Bowling (1936). *J. Dairy Sci.* **19**, 597.
(166) —— —— —— (1938). *J. Dairy Sci.* **21**, 112.
(167) Eveleth, Eveleth & Walsh (1937). *N. Amer. Vet.* **18**, 18.
(168) Haig (1935). *Proc. Ann. Meeting West Div. Amer. Dairy Sci. Ass.* **21**, 51.
(169) Duncan & Huffman (1938). *J. Dairy Sci.* **21**, 111.
(170) Moore, Sholl & Hallman (1936). *J. Dairy Sci.* **19**, 441.
(171) Seekles (1936). *Acta brev. neerl. Physiol.* **6**, 83.

636.2:612.3

II. NUTRITION

Physiology of digestion

Assmann(1) has recently studied the weights of the stomach, intestine and the alimentary contents of bullocks. He found that the empty stomach accounts for 3–5% of the body weight, the empty intestine 1·7–2·4%, the stomach contents 8·6–16·6% and the intestinal contents 1·7–5%. For comparison with non-ruminants it may be noted that in pigs the corresponding figures were 0·5–0·7, 2·5–3·2, 0·25–0·45 and 1·26–1·86%. In large bullocks the alimentary tract and contents accounted for 30% of the body weight. Swett *et al.*(2) in a very comprehensive study of the weights of the various organs found similar results.

The effect of feeding long and short hay on chewing and rumination has been investigated by Kick & Gerlaugh(3). Using three steers, 18–32 months old, with fistulae, alfalfa hay was fed uncut, 2 in. long, $\frac{1}{4}$ in. long and ground. After 24 hr. the rumen contents were removed, weighed and sampled. With the more finely ground fodder the number of chews decreased, although the younger animals needed more chews than the older. There was little difference between the uncut hay and the 2 in. sample. The shorter material gave less rumination. After 24 hr. the amount of fibre in the rumen decreased from the uncut to the $\frac{1}{4}$ in. sample but increased with the ground hay. With the two younger steers the protein in the rumen tended to diminish with the fineness of the hay. In a later paper Kick *et al.*(4) found that there was a saving in energy on grinding hay. Much more chewing energy was expended during rumination of hay alone than during that of a mixed ration. When the ration consisted of a protein supplement and maize only, the amount of rumination was reduced to a minimum with no ill effects to the animals during the 2-week period. Columbus(5) investigated the effect of different foods on the rate of passage of the food, using the dyed straw method. The rate was influenced by the physical properties of the food, fine division giving quick passage. Green fodder was more rapidly digested, but the rate of digestion was diminished with an insufficient amount of roughage. Exercise also accelerated the rate of digestion.

Analysis of the saliva of cattle, sheep, goats and pigs has shown that the amylase content is very low(6).

By a mask spirometer method, Washburn & Brody(7 8) measured simultaneously the rumen and expired gases from a cow. The ratio between the fermentation carbon dioxide and the combustible gases was subject to considerable but characteristic variations. The total energy losses in fermentation gases were 12–16% of the feed energy intake or 25–40% of the maintenance requirement. Rumen oxygen and nitrogen values showed an aerobic fermentation. As digestion proceeded the values for the rumen gases approached those of the intestinal gases.

Regarding bacterial digestion of cellulose in the fore-stomachs of ruminants Woodman & Evans(9) have made further observations from their *in vitro* studies. From previous experiments(10, 11, 12) these authors had concluded that although the normal end products of fermentation of pulped filter paper at 65° C. were organic acids and gaseous compounds, this action did not take place in the rumen but stopped at the glucose stage. In the latest series of experiments(9) using cultures of thermophiles (65° C.) and bacteria from the sheep's rumen (37° C.) they found that only traces of glucose appeared, the reaction going to the fatty acid stage. When glucose alone was fermented pyruvic acid was formed first and then reduced to lactic acid, volatile fatty acids and gaseous products. Whereas at 65° C. large amounts of lactic acid

were formed, at 37° C. only small amounts appeared. Recently Crampton & Maynard(13) have proposed new methods for the determination of cellulose and lignin in feeds and faeces. They suggest that the partition of the carbohydrates into lignin, cellulose and other carbohydrates may have more biological significance in predicting feeding values than the crude fibre and nitrogen-free extract.

Difference in digestion between sheep and cows have been found by Forbes *et al.*(14). They found that when the ratio of concentrates to fibre in the ration was 1:1 the cows digested the fibre better than sheep. When the ratio was 2:1 the sheep digested the fibre more efficiently.

The effect of acids on the digestibility of foodstuffs has been studied. Popov(15) noted that mixtures of lactic, acetic, butyric and formic acids when added to a basal ration gave an increased digestibility of proteins and fat with sheep. The acids had no effect on the pepsin and ptyalin except when the *p*H of the medium altered. He states that the acids in the silage may not be the factor responsible for the better digestion since a lowered digestibility of crude fibre was found. Kolpakow & Frühauf(16) also found a lowered digestibility of crude fibre after the addition of acid to the ration.

The factors regulating the flow of gastric juice in calves has been investigated by Espe & Cannon(17). Using three calves with abomasal Pavlov pouches they found that neither the sight of food nor sham feeding gave an increased flow of gastric juice. In the adult ruminant a psychic secretion is unnecessary since solid food passes into the rumen and is delivered into the true stomach only after some hours.

Brüggemann & Buss(18), analysing the ether extract of the abomasum fluid, have noted the presence of ether soluble organic acids or acid reacting substances. The value for the titratable acidity was higher than could be accounted for by the lactic acid present.

The effect of intestinal anastomosis on sheep has been studied by Besselaar & Quin(19). Using a side to side anastomosis, the proximal half of the small intestine was joined, in eight merino sheep, to the tip of the caecum, the lower half of the small intestine being left *in situ* after closure of the stump distal to the anastomosis. In the second series of eight sheep the proximal half of the small intestine was anastomosed to the ansa spiralis of the large intestine, thus excluding both the lower half of the small and the upper third of the colon. All the animals were kept on the same adequate ration. Following loss of condition and diarrhoea thirteen of the sixteen sheep died in 12 months. Post-mortem examination revealed varying degrees of cachectic and atropic changes, especially in those parts of the intestines excluded by the anastomosis. These parts were very anaemic and thread-like and no retrograde filling took place. As regards condition no significant difference was noted in any of the sheep. In those animals which had lost the function of the proximal third of the colon inspissated secretions but not food residues were found in the caecum.

Regarding the rapidity of digestion of fats by commercial preparations of pancreatic substance, Hartwell(20) found that, when reconstituted cream was taken as standard, coconut oil was digested most rapidly, palm kernel and castor oil next, followed by butter and other oils and fats.

Finally attention must be drawn to the book by Verzar & McDougall on absorption from the intestine(21) and that by Maynard on animal nutrition(22).

Energy metabolism

Recently Kleiber(23) has made a comprehensive survey of the efficiency of food utilization of animals. Using the total efficiency of energy utilization, the ratio of net energy in the product to total food energy, as a characterization of the process

of animal production, he found that in certain instances, such as at maintenance level or when the animal eats less than its maintenance requirement, the nutritive value of the food was zero or even negative, an assumption which he regards as unreasonable. He therefore gauges the food value *not* as the quotient of total net energy in the product and total food energy but as the quotient of a *difference* in net energy and the corresponding *difference* in food energy. Using Kellner's experiments, where the total efficiency was positive, and Armsby's, where the total efficiency was negative, he has shown that the quotient $\frac{\text{difference in net energy}}{\text{difference in food energy}}$ was positive. This quotient, characterizing the nutritive value of the food with respect to energy utilization, is called the *partial efficiency* since it represents the efficiency for a part of the food. Kleiber then proceeded to formulate mathematically the relation of total efficiency (measure for the success of animal feeding), the partial efficiency (measure for the nutritive value of the food) and the plane of nutrition (measure for the fitness of the animal and the skill of the feeder). For the special case of the partial efficiency being independent of the plane of nutrition the total efficiency of energy utilization is the difference between the partial efficiency and the reciprocal of the relative food level. The relative food level indicates how many times as much energy an animal consumes as it loses during fasting. Comparing the results of Kellner(24) and Fingerling(25), who assumed that the partial efficiency for maintenance was the same as for fattening and independent of the plane of nutrition, with those of Armsby(26), Mollgaard(27), Forbes *et al.*(28) and Wiegner(29) who noted that the partial efficiency for fattening was lower (20%) than that for maintenance, Kleiber concludes finally, from a mathematical study, that the simple formulation of Armsby and Mollgaard is correct. This is based on the assumption of a constant partial efficiency of the metabolizable food energy within maintenance and within production. He states, however, that in further determinations a standardization of the plane of nutrition is advisable. Considering the problem from the point of view of breeding, Kleiber states that the determination of the food capacity of an animal on a standard food should be one of the most important criteria with respect to energy utilization. "It is reasonable to assume that systematic breeding on the basis of relative food capacity would result in an increase in the economy of animal production." From previous work(30) Kleiber has already shown that the food capacity is essentially independent of body size but is related in the same way as the fasting catabolism or the maintenance requirement, that is to the body weight raised to the power 0·73(31).

The mathematical interpretation by Kleiber has been confirmed, in part, by the experiments of Winters(32). The results of feeding steers for 3 years have shown that there is an important variation in the efficiency of feed utilization between steers of a given grade. In the least efficient animals it cost 26–44% more to produce 100 lb. gain than in the most efficient steers. The temperature of the sheds will also affect the utilization of the food energy. Bedniagin(33) has shown that the standards in Europe for cattle in warm sheds are of little value in Russia where the sheds are cold and the outside temperature -20 to $-30°$ C. This effect is due to the heat lost by the animals which was calculated according to Rubner's law that loss of heat is proportional to body surface.

The energetic efficiency of production has been intensively studied by Brody and his co-workers during the past two years. Although in certain experiments rats have been used as experimental animals the work is of such importance that a review seems essential. Using 368 cows on 120-day tests, Brody & Cunningham(34) found that the gross energetic efficiency of milk production is independent of body weight. It is the percentage ratio of energy in the milk to energy in the digestible feed consumed. Milk was produced by Jerseys and Holsteins at the same efficiency,

34%. The *gross* energetic efficiency of good cows is of the same order as that of early post-natal growth, whereas the *net* energetic efficiency is the efficiency of lactation aside from maintenance cost and is 60%. The law of diminishing returns holds for both gross and net efficiency. The same conclusion was reached in a later study using both rats and cows(35). With respect to body weight rats produce more milk energy than cows (milk cal./B.W. in kg. = 200 for rats and 25 for cows). Using the basal metabolism or body weight raised to the power 0·73, the production for both species is the same, i.e. 2. Brody & Nisbet conclude that milk energy production is the same in all animals. Continuing this work Brody *et al.*(36) attempted to compare the basal metabolism, post absorptive, during normal lactation with the non-lactating rats. The post-absorptive metabolism could not, however, be determined during normal lactation since this was not compatible with the post-absorptive state. From the fact that the heat production of lactating rats (not post-absorptive) was double the non-lactating, Brody *et al.* state that the extrapolation methods used with humans, showing that the basal metabolism of lactating and non-lactating women is the same, may be wrong. In connexion with the energetic efficiency of milk production it is interesting to note that the *net* efficiency of egg production in hens (not counting maintenance) is of the same order as that of milk production(37). The *gross* efficiency, however, is only about one half that of milk production. The *gross* energy efficiency of muscular work also appears to be independent of body weight but varies with the basal metabolism or the body weight raised to the power 0·73(38). It is of the same order in large and small horses and in man.

An interesting paper by Brody & Cunningham(39) on the relationship of gross energetic efficiency of production and monetary profit shows that the latter increases with increasing body weight. The monetary profit per unit of body weight, however, decreases with increasing weight, while the profit per unit feed consumed is independent of body weight and is directly proportional to the energetic efficiency.

During pre-natal growth the surface law is not applicable(40). The amount of extra heat produced, Q, during gestation above the non-gestation level, when at rest, is related to the birth weight, M, of the offspring by $Q = aM^n$. n is about 1·2 and Q about 4400. The formation of a foetus of 1 kg. at birth is associated with a heat increment of gestation of 4400 cal. When the birth weight is not 1 kg., the heat increment varies with M raised to the power 1·2. In rats the resting energy metabolism, measured from the respiratory quotient, is the same during gestation and sex rest, cal. per day/(B.W.)$^{0.73}$ = constant(36). In growing cows, however, the metabolism in gestation increases more rapidly than (B.W.)$^{0.73}$. Brody *et al.* conclude that the increase in live weight without any apparent food energy cost is accounted for *not* by the lowering at the resting energy metabolism, but by (*a*) a reduction of the spontaneous physical activity with a saving of energy which is applied to body weight increase, (*b*) a saving of the specific dynamic action due to storage of protein, (*c*) the watery nature of the gestation gains, the amniotic fluid, placenta and hydration of the mother, and (*d*) the maintenance cost of the pregnant uterus being possibly nil.

Recently Gaines(41) investigated the working maintenance of animals and the relationship with live weight. By working maintenance is meant the total energy expenditure minus the milk energy and the energy required for gain in weight, i.e. the energy expenditure on basal metabolism movements and the work of elaborating and secreting milk. Gaines criticizes the theory that active tissue, for growth or production of milk, is proportional to (B.W.)$^{0.73}$. Although, from the observed inter-species relation, basal metabolism is proportional to (B.W.)$^{0.73}$ this will not be true for production. If active tissue is proportional to (B.W.)$^{0.73}$ it follows that the percentage of active tissue is related to B.W. and will vary with it. Comparing different species, cow, goat, cat, rat and mouse, this theory would lead to the conclusion that

the mouse has sixteen times as high a percentage of active tissue as a cow, while with the bacterium the absurd result of 2,000,000% active tissue is obtained. Obviously this view is untenable and Gaines concludes that in all animals the percentage of active tissue is substantially the same but that the rate of activity varies, a conclusion which would appear to be open to some of the objections brought forward for the older theory. Gaines attempts to clarify his theory by stating that, using a cow and a goat, although, under complete rest and fast the rate of activity of the active tissue of the goat is twice as rapid as in the cow, this will not hold when the minimum metabolism is superseded by the metabolism of locomotion, heavy feed consumption and heavy work connected with lactation, i.e. although basal metabolism is proportional to (B.W.)$^{0.73}$, working maintenance is not. He uses similar reasoning to establish the fact that potential milking capacity need not be proportional to (B.W.)$^{0.73}$. Discussing the importance of this problem for the determination of lactation efficiency, Gaines suggests that the upper limit of the capacity of the animal to produce milk is set by the work of energy transformation and that the possible work is proportional to the size of the animal, as measured by the initial live weight. Gaines finds support for his theory in the earlier experiments of Moulton, who found that the amount of body protein and blood was a constant percentage of live weight, and Carman & Mitchell(42), who, with white mice, showed the absurdity of considering the protoplasmic content of animals as being proportional to surface area or (B.W.)$^{\frac{2}{3}}$.

In a study of the relationship of body weight to chest girth in dairy cattle of mixed ages Brody *et al.*(43) found that the equation $Y = aX^n$ held for all practical purposes, where Y is body weight, X chest girth. "n" varied from 2·72 to 2·88, while variations in "a" were also found, showing that the nutritive level of the animal must also be considered. With an average value for "n" of 2·82 this means that the percentage increase in body weight is 2·82 times the percentage increase in chest girth.

The mode of utilization of the energy of foodstuffs in the body has been studied by Kajanoja(44). Seven cows were placed, after calving, on rations adequate in proteins, fats, etc., but inadequate in net energy for the level of production of milk. The addition of food to the ration reduced the draft on the body reserves, but not by the full amount of the added net energy. Some part of the additional energy served to increase the milk production. Diets high and low in protein, adequate and inadequate for growth, appear to have no effect on the utilization of energy in the growing rat(45). On the high protein diet the animals gained more protein, water and ash, on the low protein more fat was gained. With sheep, Watson *et al.*(46) found that the live weight increase of fattening sheep was proportional to the calories fed. The gain per pound of additional starch equivalent was 0·227 lb.

In a study of the metabolizable energy of steers and cows in 120 experimental periods, Kriss(47) found that there was a parallelism between the percentage heat lost by evaporation of water and the water consumption. The changes in the proportion of heat lost by vaporization of water follow more closely changes in the metabolizable energy than changes in the heat production while changes in the metabolizable energy gave changes in the vapour heat percentage in the same direction. Below the maintenance level the effect of the ration on the heat lost by evaporation of water is less than above maintenance. Clipping of the animals reduced but did not eliminate the effects of the plane of nutrition on the manner of heat disposal. Mitchell & Hamilton(48) also noted that the length of the hair coat of steers is a potent factor through its effect on skin temperature and indirectly on the vaporization of water from the skin. The insensible loss of weight could be lowered 20–30% by shearing. Whereas the environmental temperature, particularly above 70° F., affected the insensible loss of weight, changes in the relative humidity had no effect up to humi-

dities of 80%. In an investigation of the metabolism of goats and sheep Ritzman *et al.*(49, 50) noted that, under basal conditions, the rectal temperature and heart rate of both species are identical, 39·1° C. and 80 per min. respectively. The basal metabolism of the goat, however, was lower than that of the sheep and was the lowest of all farm animals. The metabolism of the female goat was 40% lower than that of a ewe of the same size, while the male goat was 15% lower than the ram. The authors found no explanation for this basal metabolic difference. The effect of temperature on the basal metabolism was studied by Schwabe *et al.*(51) using rats. Increasing the surrounding temperature (7–12° C.) caused an increase in the basal metabolic rate of 11–16%, increased the body temperature, retarded the gain in weight but did not affect the respiratory quotient. The increase in the basal metabolic rate could not be entirely explained by the rise in the temperature.

Mineral metabolism

(*a*) *Calcium and phosphorus*

During the past two years stress has been laid on the calcium and phosphorus requirements of ruminants and the effect of varying calcium:phosphorus ratios in the rations. Kuhlman *et al.*(52), using dairy cows over one to five lactations, found no difference in production, reproduction, health or thriftiness when the Ca:P varied from 1:1·27 to 1:1·66. Bohstedt(53) noted that the most favourable ratio was less than 2:1 and states that the majority of the protein concentrates supply a sufficient amount of phosphorus. For sheep and cattle Crampton(54) found that the minimum calcium and phosphorus requirements were the same. Expressed as a percentage of the total dry matter of the ration the requirements are: Ca 0·2–0·4%, P 0·2–0·35%. Beeson *et al.*(55) in experiments to find the optimum phosphorus necessary for the growth and fattening of lambs has shown that a fattening lamb requires 2·5 g. phosphorus daily per 100 lb. live weight for optimal gains.

The effect of excess calcium and phosphorus in the diet of sheep has been studied by Swart(56). Using twelve merino wethers divided into two groups, basal ration and basal plus very large quantities of calcium and phosphorus, he found that excessive mineral supplements retarded growth rate of body and fleece, decreased the staple length and fibre diameter but increased the extensibility of the wool. An excess of either element has, however, different results. Cox & Imboden(57), using rats, found that whereas an excess of calcium in the diet gave poor results in gestation and lactation, irrespective of the Ca:P ratio, an excess of phosphorus was very much better tolerated.

It has long been known that a phosphorus deficiency in the diet of animals leads to a reduction in the blood inorganic phosphorus. At parturition Van Landringham *et al.*(53) found that the normal fall in phosphorus was enhanced with a low phosphorus ration, while a similar fall was noted during lactation. Sienczewski(59) has also reported that the most serious symptoms of osteomalacia appear after parturition, the cows being unable to rise and becoming emaciated. Feeding young heifers and steers for 24 months on varying ratios of Ca:P Theiler *et al.*(60) found that a phosphorus deficiency leads to rickets or osteomalacia. Osteodystrophia fibrosa is not produced by a phosphorus deficiency *per se* but probably by a calcium deficiency. The Ca:P ratio, however, does not determine the onset of rickets but is associated rather with food consumption, bone growth and clinical symptoms. Stewart & Ponsford(61) have noted cases of acute pining of animals associated with infestation with nematode parasites. In the chronic stage this infestation is insufficient to account for the degree of anaemia and emaciation found. Analysis of the pasture

showed a low content of calcium, phosphorus and iron, and the authors state that the combined effects of malnutrition and gastro-intestinal parasites give the resultant condition. With pigs Aubel *et al.*(62) found that a phosphorus deficiency leads to a lowering of the blood inorganic phosphorus, failure of normal growth and development of bone and muscle, poor utilization of feed and storage of energy, loss of appetite and increased thirst and urinary excretion. The kidneys show signs of chronic diffuse nephritis of the parenchymatous type. A low calcium diet with an abnormal Ca:P ratio has been found to lead only to osteoporosis and a lower ash content of the bones, according to Theiler *et al.*(63). No change was noted in the phosphatase content of the blood. A low calcium and phosphorus ration with a normal Ca:P ratio only slowed down the growth rate and gave poor food consumption. No severe symptoms were noted at any time.

The effect of different types of calcium and phosphorus supplements has also been studied. Arnold & Becker(64) found that the addition of bone meal as a 2% supplement of the concentrate ration containing low calcium roughage gave an increase of 45% in the milk yield. The animals on the unsupplemented ration showed a more rapid decrease in milk production. The results of the experiments of Newlander *et al.*(65) show that, supplementing a basal ration with either commercial dicalcium phosphate or steamed bone flour, both forms are equally valuable as regards assimilability of the calcium and phosphorus. The tricalcium phosphate, on the other hand, appears to be better assimilated than the dicalcium whether or not allowance was made for the excess milk secreted(66). When, however, allowance was made for both the excess milk produced and the excess intake there was no significant difference between the two forms. With sheep Godden & Ray(67) found that dicalcium phosphate was utilized to a greater extent (50%) than steamed bone flour and gave a more favourable influence on nitrogen assimilation. Kleiber *et al.*(68) also found dicalcium phosphate valuable in correcting a phosphorus deficient ration. The cessation or irregularity of oestrous found with the phosphorus deficiency became rapidly normal. Similar results were obtained by Maynard *et al.*(69) with steamed bone flour. Green(70) found that the mono-calcium phosphate was assimilated better than bone meal. The appetite of the cattle for the mono-calcium salt, however, varied with the season, age and condition of the cattle and with the feeding and management.

The effect of the addition of acids, sulphuric or hydrochloric acids at the rate of 10 equivalents per 100 kg., to silage with calcium carbonate has been studied by Kirsch & Gramatzki(71). The acids increased the feeding value of the silage, giving, with sheep, positive calcium and phosphorus balances.

Ayyar(72) has studied the effect of fodders at different stages of maturity when fed to cattle. The early cut material gave a high urinary output due to the high alkali content of the food. As maturity advanced the total bases decreased. He found that the species of grass and the stage of maturity affected the mineral supply to the animal and the acid base balance. The urinary excretion of calcium and magnesium depended on the *p*H of the urine. There was an inverse relationship between the urinary calcium and phosphorus. Brull(73) found that the level of the plasma phosphorus governs the urinary excretion, for which a definite threshold exists. This was controlled by the endocrine glands and was affected by narcosis and renal poisons. Above certain levels of phosphorus in the plasma a colloidal calcium-phosphorus complex forms which cannot be excreted. The urinary phosphorus is only slightly affected by diuresis. Brull's evidence seems to show that phosphorus is eliminated by glomerular filtration and by tubular secretion.

Lactose has a favourable influence on the utilization of calcium and phosphorus in immature but not adult animals(74). This was found to be true at all levels of calcium and phosphorus. The calcium excretion into the gut was significantly dimin-

ished by the addition of lactose to the ration. With radioactive phosphorus Chievitz & Hevesy(75) found that, in humans, 15–20% of the faecal phosphorus had been re-excreted into the gut via the digestive juices. In rabbits, Hahn *et al.*(76) noted that the amount re-excreted was 11·5%. The average time spent by the phosphorus atom in the body is 30 days. Within 27 days 45% of the labelled phosphorus was excreted through the kidney. The ratio of active to normal phosphorus was highest in the kidney muscle and liver and lowest in the bones.

Recently Westerlund(77) made a survey of the early experiments of Forbes and Perkins & Munroe. His analyses of the results yield the following conclusions: (*a*) the factors influencing the faecal calcium output are intake of calcium, phosphorus, digestible protein and milk yield, (*b*) an increase in the calcium intake gives an increased faecal calcium, (*c*) increased phosphorus intake gives reduction in faecal calcium, (*d*) varying intakes of digestible protein influence the net calcium absorption, (*e*) heavy milkers absorb more calcium than light milkers, (*f*) there is no constant optimum value for the Ca:P ratio.

Carbery *et al.*(78) have recently shown that with rice straw the calcium requirement is high, due possibly to the high potassium intake or to the presence of an anti-calcifying agent. Heavy potassium ingestion gives a heavy calcium excretion even to the point of a negative balance. The requirements per 500 lb. live weight, using paddy straw as roughage, are 24 g. CaO and 10 g. P_2O_5.

(*b*) *Other minerals*

Mulhearn(79) found that an intrajugular injection of either magnesium chloride or calcium chloride cured grass tetany. A drench of magnesium sulphate had the same effect. According to Cunningham(80) this is not due to a deficiency of magnesium. The magnesium content of the pastures on which the cows suffered was not low, neither was the magnesium content of the bones and organs. He concludes that there may be a temporary impairment of magnesium absorption.

The effect of fluorine on the animal body has been studied by several workers. Elmslie(81) found that feeding rock phosphate for $5\frac{3}{4}$ years, in which the fluorine intake was 1·2–2·0 mg./kg. body weight, had no effect on growth, appetite, milk production or condition of the animals. The weight of the calves was slightly less and the teeth became abraided. Elmslie concludes that the toxic level of fluorine intake lies between 1·5 and 3 mg./kg. body weight daily. Du Toit *et al.*(82) found that appreciable amounts of fluorine are retained by cattle. When receiving 60 mg. daily they retained 12–18%. Only 9% was excreted in the urine and about 82% in the faeces. With massive amounts of fluorine, 738 mg. daily, 34% was retained and 40% of the total excretion found in the urine. Fluorine ingestion gave a phosphorus deficiency and osteomalacia. Evans *et al.*(83) examined the fluorine content of the bones of calf embryos (2–10 weeks), foetuses (4–9 months), young calves and mature cows. The fluorine content of the embryos was 37 p.p.m. (18–60), foetuses 30 p.p.m., young calves 32 p.p.m. and mature cows 190 p.p.m. Apparently a small amount of fluorine is transferred through the placenta of the cow to the foetus. During suckling the fluorine storage in the bones of young calves is low, showing a minimum secretion of fluorine in the milk.

Malan *et al.*(84) found, in a controlled experiment using 40 ewes, that the addition of 0·05 g. potassium iodide to the ration caused a heavy death rate among the lambs 3 days after birth. With flowers of sulphur(85) no effect was noted on body weight, food consumption or wool production after 13 months.

An interesting case of salt poisoning of a cow grazing on a salt marsh has been reported by Battaglino(86). The animal was only able to walk, showed intense thirst,

staggered, had dilated pupils, a rapid pulse and muscular tremors. Recovery followed after injection of caffeine and an unlimited supply of fresh water.

Finally attention may be drawn to four review articles on mineral nutrition and deficiencies which have appeared within the last two years(87–90).

(*c*) *Vitamins*

Vitamin A. Using cows, calves, ewes, lambs and pigs, Guilbert *et al.*(91) found that, using carotene in alfalfa or in solution in oil as a source of vitamin A, the minimum requirement was 25 μg./kg. body weight per day. It was the same for all three species, whether growing, adult or pregnant. The corresponding value for vitamin A in cod-liver oil was 6–8 μg. They regard an amount 5–10 times this physiological minimum as the practically desirable minimum. With calves, the addition of vitamin A to a ration consisting of partially skimmed milk, alfalfa hay and grain gave a slight but non-significant increase in growth in a 40-week trial(92). Hart & Miller(93) studied the effect of an A-avitaminosis with sheep. A diet low in vitamin A when given to adult ewes for 5 months, including the breeding season, did not affect the number and health of the lambs, although the ewes lost weight at first. Where a low vitamin A diet was fed to the young ewes and continued through gestation all but one lamb died soon after birth. A further depletion gave night blindness at the time of breeding and none of the lambs lived. Recently a short review of the effects of vitamin A deficiency has appeared(94). The symptoms are (*a*) cessation of growth, (*b*) xerophthalmia, (*c*) nyctalopia or night blindness, (*d*) susceptibility to infection in the alimentary, respiratory and urino-genital tract, (*e*) delaying of sexual maturity and infrequency of ovulation, and (*f*) demyelination of tracts in the spinal cord.

The decrease in the vitamin A potency of feeds has been studied by Fraps *et al.*(95, 96). With a ration consisting of alfalfa hay the potency decreased by 7·5% per month, while the potency of the butter fat also declined from 40 to 60 Sherman units per g.(95). When cod-liver oil is added to a ration and stored the vitamin A is completely lost(96) even in the presence of a stabilizer such as hydroquinone. Carotene is very much more stable in rations, although most is lost if the feed is not used within two to three months. Hauge *et al.*(97) found that there is a factor present in soya beans which suppresses the transference of vitamin A activity from feed to milk fat. This factor is present in the oil and fat extracted residue. Prolonged extraction of the soya bean with alcohol did not remove the factor.

Vitamin D. Krupski & Almasy(98) have reported on two rachitic cows over a period of three years. They found no improvement in condition by putting the cattle to pasture from spring to autumn. The urinary phosphorus of the rachitic animals was 47—67 times that of normal animals, and the serum phosphate was also abnormally high. Large doses of vitamin D decreased the urinary and serum phosphorus and increased the blood phosphorus. The first symptom of a vitamin D deficient diet is, according to Wallis(99), a lowering of the serum and inorganic phosphorus. Balance trials showed a loss of calcium and phosphorus. Loss of appetite was found together with enlargement of the joints and humping of the back. The ration fed consisted of molasses beet pulp, corn, oats, corn gluten meal, bone mean and vitamin A.

In dairy calves a vitamin D deficiency is shown by (*a*) progressive downward trend in plasma calcium and inorganic phosphorus, (*b*) anorexia, (*c*) a decrease in the growth rate and (*d*) stiffness, bowing of the legs and a reduction of the rib ash(100). In cattle more than 190 days old Bechtel *et al.*(101) found that maize silage, supplying 122–165 U.S.P. units of vitamin D per lb. dry matter, prevented the onset of rickets and supported normal growth and reproduction. Under 190 days maize silage was of little value due to anorexia and the low intake of silage. Bechdel *et al.*(102) studied

the vitamin D requirements of calves from birth to 6 months. Thirty-three Holstein calves were used, kept in dark stables and confined to separate stalls. Whole milk was fed for 4 weeks and skim milk up to 4 months, together with a commercial preparation of carotene. To different groups activated yeast and cod-liver oil were given. In 4–5 months the control animals were rachitic, whereas yeast and cod-liver oil were equally effective in prevention. The vitamin D requirement was 300 U.S.P. units per 100 lb. live weight from birth to 6 months.

Vitamin B. The effect of a vitamin B deficiency has been studied on sheep. Pallaske(103) noted a disease affecting lambs, after poor winter feed, similar to that affecting pregnant sheep. He suggested a multiple deficiency of vitamins A and B_1. The principal lesions were nervous but the mucous membrane was also affected. In ewes Bendinger & Rjanitzina(104) found a disease which is primarily a derangement of the nervous system. Only adult ewes were affected, and only at the end of the winter, the mortality rate being 100%. The authors claim that it is a case of a vitamin B deficiency.

Recently Dubois(105) has suggested that there exists a relationship between the vitamins themselves and between the vitamins, minerals and endocrine glands. In any diet there should be (*a*) a complex containing all the vitamins in definite proportions, (*b*) a mixture of minerals in definite amounts and (*c*) diastase. He states that unless this is carried out any additions may be ineffective.

Finally attention must be drawn to reviews on vitamins which have appeared during the last two years(106, 109).

S. MORRIS

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REFERENCES

(1) ASSMANN (1937). *Z. Fleisch- u. Milchhyg.* **47**, 480.
(2) SWETT, MATTHEWS, MILLER & GRAVES (1937). *Bur. Dairy Ind.*, U.S. Dep. Agric., B.D.I.M. 589.
(3) KICK & GERLAUGH (1936). *Proc. Amer. Soc. An. Prod.* p. 93.
(4) KICK, GERLAUGH, SCHALK & SILVER (1937). *J. agric. Res.* **55**, 587.
(5) COLUMBUS (1936). *Forschungsdienst.* **2**, 208.
(6) MATSUOKA (1937). *J. agric. Chem. Soc. Japan*, **13**, 872.
(7) WASHBURN & BRODY (1937). *Res. Bull. Mo. agric. Exp. Sta.* no. 263.
(8) WASHBURN (1937). *J. Dairy Sci.* **20**, 407. *Proc.*
(9) WOODMAN & EVANS (1938). *J. agric. Sci.* **28**, 43.
(10) WOODMAN (1930). *Biol. Rev.* **5**, 273.
(11) WOODMAN & STEWART (1928). *J. agric. Sci.* **18**, 713.
(12) —— —— (1932). *J. agric. Sci.* **22**, 527.
(13) CRAMPTON & MAYNARD (1938). *J. Nutrit.* **15**, 383.
(14) FORBES, BRATZLER, BLACK & BRAMAN (1937). *Bull. Pa agric. Exp. Sta.* no. 339.
(15) POPOV (1936). *Ann. Univ. Sofia.* **5**. *Fac. agron. sylvic.* **14**, 93.
(16) KOLPAKOW & FRÜHAUF (1937). *Forschungsdienst.* **3**, 170*R*.
(17) ESPE & CANNON (1937). *Amer. J. Physiol.* **119**, 720.
(18) BRÜGGEMANN & BUSS (1937). *Arch. wiss. prakt. Tierheilk.* **72**, 353.
(19) BESSELAAR & QUIN (1935). *Onderstepoort J. Vet. Sci.* **5**, 501.
(20) HARTWELL (1938). *Biochem. J.* **32**, 462.
(21) VERZAR & MCDOUGALL (1936). *Absorption from the Intestine.* Longmans, Green and Co.
(22) MAYNARD (1937). *Animal Nutrition.* McGraw-Hill Book Co.

(23) KLEIBER (1935). *Conference on Energy Metabolism.* Pennsylvania.
(24) KELLNER (1900). *Landw. Versuchsw.* **53**, 463.
(25) FINGERLING (1914). *Landw. Versuchsw.* **84**, 149.
(26) ARMSBY (1922). *Nutrition of Farm Animals*, p. 361.
(27) MOLLGAARD (1929). *Futterungslehre des Milchviehs*, p. 118.
(28) FORBES (1926). *J. agric. Res.* **33**, 492.
(29) WIEGNER (1930). *Tierernährung*, **2**, 193.
(30) KLEIBER (1932). *Hilgardia*, **6**, 315.
(31) BRODY (1934). *Res. Bull. Mo. agric. Exp. Sta.* no. 220.
(32) WINTERS (1936). *Proc. Amer. Soc. An. Prod.* p. 263.
(33) BEDNIAGIN (1935). *J. Sci. Comm. Kazakstan Gov.* **1–2**, 11.
(34) BRODY & CUNNINGHAM (1936). *Res. Bull. Mo. agric. Exp. Sta.* no. 238.
(35) BRODY & NISBET (1938). *Res. Bull. Mo. agric. Exp. Sta.* no. 285.
(36) BRODY, RIGGS, KAUFMAN & HERRING (1938). *Res. Bull. Mo. agric. Exp. Sta.* no. 281.
(37) BRODY, FUNK & KEMPSTER (1938). *Res. Bull. Mo. agric. Exp. Sta.* no. 278.
(38) BRODY & CUNNINGHAM (1936). *Res. Bull. Mo. agric. Exp. Sta.* no. 244.
(39) —— —— (1936). *Res. Bull. Mo. agric. Exp. Sta.* no. 244.
(40) BRODY (1938). *Res. Bull. Mo. agric. Exp. Sta.* no. 283.
(41) GAINES (1937). *J. Dairy Sci.* **20**, 583.
(42) CARMAN & MITCHELL (1936). *Amer. J. Physiol.* **76**, 380.
(43) BRODY, DAVIS & RAGSDALE (1937). *Res. Bull. Mo. agric. Exp. Sta.* no. 262.
(44) KAJANOJA (1937). *Biederm. Zbl.* B **9**, 74.
(45) JOHNSON, HOGAN & ASHWORTH (1936). *Res. Bull. Mo. agric. Exp. Sta.* no. 246.
(46) WATSON, SKILBECK & ELLIS (1937). *Emp. J. exp. Agric.* **5**, 180.
(47) KRISS (1936). *Amer. J. Physiol.* **116**, 262.
(48) MITCHELL & HAMILTON (1936). *J. agric. Res.* **52**, 837.
(49) RITZMAN, WASHBURN & BENEDICT (1936). *Tech. Bull. N.H. agric. Exp. Sta.* no. 66.
(50) —— —— —— (1936). *Tech. Bull. N.H. agric. Exp. Sta.* no. 66.
(51) SCHWABE, EMERY & GRIFFITH (1938). *J. Nutrit.* **15**, 199.
(52) KUHLMAN, WEAVER, GALLUP & NALBANDOV (1936). *Proc. Amer. Soc. An. Prod.* p. 55.
(53) BOHSTEDT (1937). *J. Dairy Sci.* **20**, 405, *Proc.*
(54) CRAMPTON (1937). *Sci. Agric.* **18**, 38.
(55) BEESON, BOLIN & HICKMAN (1937). *Proc. Amer. Soc. An. Prod.* p. 245.
(56) SWART (1937). *Z. Züchtung B*, **37**, 77.
(57) COX & IMBODEN (1936). *J. Nutrit.* **11**, 147.
(58) VAN LANDRINGHAM, HENDERSON & BOWLING (1936). *J. Dairy Sci.* **19**, 597.
(59) SIENCZEWSKI (1936). *Wiad. weteryn.* **18**, 67.
(60) THEILER, DU TOIT & MALAN (1937). *Onderstepoort J. Vet. Sci.* **8**, 375.
(61) STEWART & PONSFORD (1936). *J. comp. Path.* **49**, 49.
(62) AUBEL, HUGHES & LIENHARDT (1936). *J. agric. Res.* **52**.
(63) THEILER, DU TOIT & MALAN (1937). *Onderstepoort J. Vet. Sci.* **9**, 127.
(64) ARNOLD & BECKER (1936). *J. Dairy Sci.* **19**, 257.
(65) NEWLANDER, ELLENBERGER & JONES (1936). *Bull. Vt agric. Exp. Sta.* no. 406.
(66) —— —— —— (1936). *J. Dairy Sci.* **19**, 443, *Proc.*
(67) GODDEN & RAY (1938). *Emp. J. exp. Agric.* **6**, 79.
(68) KLEIBER, GOSS & GUILBERT (1936). *J. Nutrit.* **12**, 121.
(69) MAYNARD, GREAVES & SMITH (1936). *Bull. Utah agric. Exp. Sta.* no. 265.
(70) GREEN (1936). *Circ. Mont. agric. Exp. Sta.* no. 148.
(71) KIRSCH & GRAMATZKI (1936). *Z. Züchtung, B*, **36**, 101.
(72) AYYAR (1935). *Ind. J. Vet. Sci.* **5**.
(73) BRULL (1936). *Ann. Physiol. Physicochem. biol.* **12**, 635.
(74) FRENCH & COWGILL (1937). *J. Nutrit.* **14**, 383.

(75) Chievitz & Hevesy (1937). *Det. kgl. Danske Vidensk.* **13.**
(76) Hahn, Hevesy & Lundsgaard (1937). *Bioch. J.* **31**, 1705.
(77) Westerlund (1937). *Lantbrukshögsk. Ann.* **4**, 55.
(78) Carbery, Chatterjee & Talapatra (1937). *Ind. J. vet. Sci.* **7**, 155.
(79) Mulhearn (1936). *Queensland Agric. J.* **45**, 44.
(80) Cunningham (1936). *N.Z. J. Sci. Tech.* **18**, 424.
(81) Elmslie (1936). *Proc. Amer. Soc. An. Prod.* p. **44.**
(82) Du Toit, Smuts & Malan (1937). *Onderstepoort J. Vet. Sci.* **8**, 359.
(83) Evans, Philips & Hart (1938). *J. Dairy Sci.* **21**, 81.
(84) Malan, Du Toit & Groenewald (1937). *Onderstepoort J. Vet. Sci.* **7**, 523.
(85) Du Toit, Malan, Groenewald & Botha (1936). *Onderstepoort J. Vet. Sci.* **7**, 181.
(86) Battaglino (1935). *Nuovo Ercol.* **40**, 288.
(87) Mitchell & McClure (1937). *Bull. Nat. Res. Coun.* (*U.S.A.*), no. 99.
(88) Dryerre (1936). *Vet. J.* **92**, 163.
(89) Mitchell (1936). *Vet. Med.* **31**, 66.
(90) *Scient. Rep. Imp. Inst. Agric. Res.*, Pusa, 1934–5.
(91) Guilbert, Miller & Hughes (1937). *J. Nutrit.* **13**, 543.
(92) Dahlberg & Maynard (1937). *J. Dairy Sci.* **20**, 59.
(93) Hart & Miller (1937). *J. agric. Res.* **55**, 47.
(94) Annotation (1937). *Austr. Vet. J.* **13**, 162.
(95) Fraps, Copeland, Treichler & Kemmerer (1937). *Bull. Texas Agric. Exp. Sta.* no. 536.
(96) Fraps & Kemmerer (1937). *Bull. Texas Agric. Exp. Sta.* no. 557.
(97) Hauge, Wilbur & Hilton (1937). *J. Dairy Sci.* **20**, 87.
(98) Krupski & Almasy (1936). *Schweiz. landw. Mh*, **12**, 285.
(99) Wallis (1937). *J. Dairy Sci.* **20**, 434. *Proc.*
(100) Duncan & Huffman (1936). *J. Dairy Sci.* **19**, 291.
(101) Bechtel, Huffman, Duncan & Hoppert (1936). *J. Dairy Sci.* **19**, 359.
(102) Bechdel, Hilston & Guerrant (1937). *J. Dairy Sci.* **20**, 432. *Proc.*
(103) Pallaske (1936). *Arch. wiss. prakt. Tierheilk.* **70**, 278.
(104) Bendinger & Rjanitzina (1935). *Sovyet Vet.* **3**, 37.
(105) Dubois (1937). *C.R. V^e Cong. inter. tech. chim. Inst. agric. Scheveningen, Holland*, **1**, 79.
(106) Butz (1936). *Z. Schweinez*, **43**, 213.
(107) Hart & Guilbert (1937). *J. Amer. vet. Med. Ass.* **91**, 193.
(108) Maynard (1937). *Cornell Vet.* **27**, 122.
(109) Schoch (1937). *Rapport Spécial XVIIIème Congrès Intern. Agric.* Sect. 6.

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